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Scavenger receptor cysteine-rich proteins on the cross-roads between innate and adaptive immunity

Carine Machado Gonçalves

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“Eu sei que o meu trabalho é uma gota no oceano, mas sem ele o oceano seria menor”

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There is no greater joy, than learn something new and interesting

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James JR, McColl JT, Oliveira MI, Dunne PD, Huang E, Jansson A, Nilsson P, Sleep DL, **Gonçalves CM**, Fernandes RA, Mahen R, Carmo AM, Klenerman D, Davis SJ (2011) Key elements of the T-cell receptor triggering apparatus are monovalent or monomeric on resting T-cells. *J. Biol. Chem*., 16;286(37):31993-2001

Oliveira MI, **Gonçalves CM**, Pinto M, Fabre S, Santos AM, Lee SF, Castro MA, Nunes RJ, Barbosa RR, Parnes JR, Yu C, Davis SJ, Moreira A, Bismuth G, Carmo AM (2012) CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. *Eur. J. Immunol.* 42(1):195-205

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ABSTRACT

Deciphering how a T cell is activated has been a major challenge to immunologists. Involving different types of cells and/or proteins, the symphony of their interactions provides the impulse for signaling pathway's initiation giving rise to an effective immune response. With the present work we intended to extend the characterization of the Scavenger Receptor Cystein-Rich (SRCR) superfamily and the role of SRCR proteins in T cell biology and T cell activation. Proteins belonging to the SRCR family share domains homologous to the membrane-distal region of macrophage scavenger receptor I and are particularly well conserved between species. These domains can be found in the extracellular regions of membrane proteins and in secreted glycoproteins, from the most primitive species to vertebrates. While SRCR proteins have common traits and similar structures, their biological role is still not clearly-defined. In order to deepen our understanding of the biology of SRCR members in T cells, we have cloned and studied a new family member that we named Soluble Scavenger with 5 domains (SSc5D), expressed in T lymphocytes. We additionally found that SSc5D is also highly expressed in monocytes/macrophages, and is enriched in placenta. The mature polypeptide is predicted to consist of 1573 amino acids comprising, towards the N-terminus, five very similar SRCR domains that are highly conserved among non-marsupial mammals, and a large (>250 nm), very heavily glycosylated, mucin-like sequence towards the C-terminus. A shorter isoform encoded by a weakly expressed, alternatively spliced transcript, which lacks the mucin-like C-terminal region, was also identified. It seems likely that SSc5D has a role at the interface between adaptive and innate immunity, or in placental function. Another member of the SRCR family, one that had a described role in T cell signaling, is CD6. CD6 is a membrane-associated glycoprotein expressed on T cells, is described to be involved in the regulation of T cell development and activation, and as having a relevant role in the context of inflammatory responses and cellular expansions as well. Contradicting the role commonly accepted for CD6, our results showed that expression of CD6 in activated T cells contributes to a significant reduction in early and late T cell responses upon superantigen presentation, or TCR triggering by mAbs. Calcium signals and IL-2 release were diminished in T cells expressing CD6, compared with CD6 negative cells. Also, proliferation of blood T lymphocytes was increased when the CD6-CD166 interaction was blocked and CD6 was dispersed throughout the entire cell surface. These data suggest that CD6 can be a signaling attenuator whose level of expression establishes the strength of signaling. As possible effectors of CD6 signaling are the Src kinases Lck and Fyn, we were interested in

studying the interaction of these kinases with CD6, measuring the binding stoichiometries at the T cell surface by using Bioluminescence Resonance Energy Transfer (BRET), which allows the organization of proteins to be studied *in situ*. Unfortunately, for a number of unexpected experimental reasons and for structural constraints of the proteins, data for the specific associations of CD6 with Lck and Fyn were not obtained. Nevertheless, we were able to determine by BRET that both Lck and Fyn are monomers, whereas the co-receptor CD4, known to associate with Lck, exhibited a tendency to self-associate. However, by co-expressing Lck with CD4 in 293T cells, we determined that CD4 behaved as a functional monomer and likely remains so at the cell surface. When bound to Lck the CD4 cytoplasmic domain has a defined tertiary structure, including an amphipatic α-helix, which explains the lack of oligomerization in the presence of Lck. Moreover, using Jurkat cells expressing different Lck mutants, we show that Lck-association with lipid rafts dictates the capacity of CD2, another Lck-binding receptor, to target to these membrane-signaling platforms. As CD2 physically interacts with both Lck and Fyn, preferentially inside lipid rafts, and reflecting the increase of CD2 in lipid rafts following activation, CD2 can mediate the interaction between the two kinases and the consequent boost in the kinase activity in lipid rafts. These results suggest that the organization and regulation of the triggering apparatus of the T cell is simpler than envisaged so far as each element is monomeric in resting cells and that translocation of proteins to specific microdomains within the plasma membrane results from protein-protein interactions induced upon T cell activation.

SUMÁRIO

Decifrar a forma como a célula T é ativada tem sido um dos maiores desafios enfrentado pelos imunologistas. O envolvimento de diferentes tipos de células e/ou proteínas e a harmonia das suas interações proporcionam o arranque para a iniciação das vias de sinalização originando uma resposta imune efetiva. O intuito deste trabalho é completar a caracterização da superfamília de recetores *scavenger* ricos em cisteínas (SRCR) e o seu papel na biologia e ativação das células T. As proteínas que pertencem a esta família são particularmente conservadas entre espécies e partilham a presença de domínios homólogos ao domínio mais afastado da membrana do recetor de macrófagos do tipo I. Estes domínios podem ser encontrados na região extracelular de proteínas de membrana ou em glicoproteínas segregadas, desde as espécies mais primitivas até aos vertebrados. Enquanto as proteínas SRCR partilham características comuns e estruturas semelhantes, a sua função biológica ainda não está bem definida. De forma a aprofundar o nosso conhecimento na biologia dos membros SRCR nas células T, clonámos e estudámos um novo membro da família que foi chamado de SSc5D (*scavenger* solúvel com 5 domínios) expresso em linfócitos T. Também foi observado que o SSc5D tinha elevada expressão em monócitos/macrófagos sendo especialmente expresso em placenta. A proteína prevista tem cerca de 1573 aminoácidos, consistindo numa região N-terminal com 5 domínios *scavenger* muito semelhantes que são bastante conservados entre mamíferos não-marsupiais, apresentando na região C-terminal uma enorme cauda citoplasmática (>250 nm), muito glicosilada e cuja sequência é semelhante à das mucinas. Foi também identificada uma isoforma mais curta, codificada por um transcrito menos expresso e que sofre de *splicing* alternativo, tendo a ausência da região C-terminal glicosilada. Parece-nos bastante provável que o SSc5D tenha uma função na interface entre a imunidade inata e adquirida. Outro membro pertencente à família dos SRCR, que já tinha sido descrito como tendo um papel na sinalização da célula T, é o CD6. O CD6 é uma glicoproteína de membrana expressa em células T, e está envolvido na regulação e ativação do desenvolvimento das células T, podendo ter um papel relevante ao nível das respostas inflamatórias assim como na expansão celular. Contradizendo o papel geralmente aceite para o CD6, os nossos resultados mostram que a expressão do CD6 em células T ativadas contribui para uma redução significativa das respostas imediata e tardia de células T ativadas, tanto com superantigénio como com anticorpos monoclonais, que desencadeiam a ativação do TCR. Observou-se a diminuição dos sinais de cálcio e da libertação de IL-2 em células T que expressavam o CD6, comparativamente com células cuja expressão do CD6 era negativa. Além disso, a

proliferação de linfócitos T isolados do sangue aumentava quando a interação do CD6 com o CD166 era bloqueada e o CD6 se encontrava disperso ao longo de toda a superfície da célula. Estes dados sugerem que o CD6 pode funcionar como um atenuador da sinalização cujos níveis de expressão estabelecem a intensidade do sinal. Os possíveis efectores que contribuem para a sinalização do CD6 são as cinases Lck e Fyn, daí o nosso interesse em estudar a interação destas cinases com o CD6, medindo a capacidade de ligação na superfície da célula T, usando BRET, um método que permite estudar a organização das moléculas *in situ*. Infelizmente, por uma série de razões experimentais e por constrangimentos estruturais das proteínas, foi impossível obter resultados específicos para demonstrar a associação do CD6 com Lck e Fyn. No entanto, conseguimos demonstrar por BRET que ambas as cinases Lck e Fyn comportam-se como monómeros, enquanto o CD4, um co-recetor intimamente ligado ao Lck, parece ter uma tendência para formar dímeros. Contudo, quando co-expressamos o Lck com CD4 em células 293T, determinámos que o CD4 comporta-se funcionalmente como um monómero e permanece desta forma na superfície da célula. Quando o Lck se liga CD4, o domínio citoplasmático do CD4 adquire uma estrutura terciária, incluindo uma cadeia hélice-α anfipática, o que poderá explicar a ausência de oligomerização na presença do Lck. Além disso, usando uma linha celular Jurkat a expressar diferentes mutantes do Lck demonstrou-se qua a associação do Lck com *lipid rafts* dita a capacidade do CD2, outro recetor que se liga ao Lck, de se localizar nestas plataformas de sinalização associadas à membrana. Como o CD2 se associa fisicamente com ambas as cinases Lck e Fyn, preferencialmente nos *lipid rafts*, poder-se-á dizer que o CD2 serve de mediador da interação de ambas as cinases e consequentemente impulsiona a atividade das cinases nos *lipid rafts*. Estes resultados sugerem que a organização e a regulação dos fatores que despoletam a ativação da célula T são simples prevendo que cada elemento é monomérico em células não ativadas e que a localização de proteínas em domínios específicos da membrana plasmática é maioritariamente devida a interações entre proteínas induzidas após ativação das células T.

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ABBREVIATIONS

Ab	Antibody
ADAP	adhesion- and degranulation-promoting adaptor protein
Ag	Antigen
AICD	Activation induced cell death
APC	Antigen presenting cell
BCR	B cell receptor
Bp	Base pair
BRET	Bioluminescence resonance energy transfer
BSA	Bovine Serum Albumin
C-terminal	Carboxy-terminal
Ca^{2+}	Intracellular free calcium
CARD	Caspase activation and recruitment domains
CLRs	C-type lectin receptors
Cbp	Csk binding protein
CD2BP2	CD2-binding protein
CDR	Complementarity determining region
CK2	Casein kinase II
Csk	C-terminal Src kinase
cSMAC	Central supramolecular activation cluster
DAG	1,2-diacylglycerol
DAMPs	Damage-associated molecular patterns
DIG	Detergent-insoluble glycolipid domain
dSMAC	Distal supramolecular activation cluster
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F-actin	Filamentous-actin
FRET	Fluorescence resonance energy transfer
GPI	Glycosylinositolphosphate
Grb2	Growth factor receptor-bound protein 2
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible costimulator
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IL-2	Interleukin-2
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine based activation motif

ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-switch motif
Itk	IL-2-inducible T cell kinase
LAT	Linker for activation of T cells
LFA-1	Leukocyte function-associated antigen 1
Luc	Luciferase
mAb	Monoclonal antibody
MAPKs	Mitogen-activated protein kinases
MHC	Major Histocompatibility complex
MTOC	Microtubule-organizing center
NFAT	Nuclear factor for activated T cells
NF-κB	Nuclear factor-κB
NK	Natural killer
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domains
N-terminal	Amino-terminal
PAG	Phosphoprotein associated with glycosphingolipid-enriched microdomains
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell(s)
PHA	Phytohemagglutinin
PIP2	Phosphatidylinositol-4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PLCγ1	Phospholipase C-γ
pMHC	Peptide-MHC complex
PRRs	Pattern recognition receptors
pSMAC	Peripheral supramolecular activation cluster
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
pTyr	Phosphotyrosine
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
SCID	Severe-combined immunodeficiency
SFK	Src family kinase
SH1/2/3	Src homology 1/2/3
SHP-1	SH2 domain-containing protein tyrosine phosphatase 1
SLAM	Signaling lymphocyte activation molecule
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
Sos	Son of sevenless
SRCR	Scavenger receptor cysteine rich
Syk	Spleen tyrosine kinase

TCR	T cell receptor
TIRF	Total internal reflection fluorescence
TLRs	Toll-like receptors
TM	Transmembrane
TRAP	Transmembrane adaptor protein
TNF	Tumor necrosis factor
TWEAK	TNF-like inducer of apoptosis
WT	Wild-type
ZAP-70	Zeta-chain-associated protein

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Chapter I

General Introduction

AIMS OF THE THESIS

Protein families are groups of evolutionary-related proteins displaying similar structures and many times having related biological functions. For example, proteins of the immunoglobulin superfamily (IgSF) have typical 3-D folds identical to extracellular domains of immunoglobulins and are usually involved in cell-cell recognition and communication in complex systems such as the immune and nervous systems; on the other hand, members of the tumor necrosis factor (TNF) superfamily are distributed into TNF ligands and receptors, the latter being typically oligomeric type I or type III transmembrane proteins containing extracellular cysteine-rich domains and intracellular death domains, while the former are type II transmembrane proteins whose extracellular domains can be cleaved to generate soluble cytokines. Although scavenger receptor cysteine-rich (SRCR) proteins are structurally very well conserved from sponges to vertebrates, no common function or unifying role has been disclosed thus far. Resulting from this, SRCR proteins are often not even considered as a group having defined functions, despite some of its members having been (individually) assigned with a given function: regardless that many SRCR proteins have a described role in the recognition of pathogen patterns, usually only C-type lectin receptors (CLRs), Toll-like receptors (TLRs) and membrane receptor kinases are viewed as major pattern recognition receptors (PRRs). Also, when addressing receptors with inhibitory roles in leukocyte signaling, killer-cell immunoglobulin-like receptors (KIRs), leukocyte inhibitory receptors (LIRs) and Ly49 homodimers receive the most attention, while some SRCR members, although embodied with a clear inhibitory role (for example CD5, and as presented in this thesis, CD6), do not make the charts and be considered as an inhibitory group in their own right.

In its origin, this thesis was designed to address the biological role of SRCR proteins in T lymphocytes. Apart from the well-known cell surface antigens CD5 and CD6, we had gathered evidence that a novel gene, which we later termed *SSc5D*, was the only additional SRCR member to be expressed in T cells. We had also preliminary but convincing evidence that CD6 could play a different role from what it was generally assumed to be. Nevertheless, our own published studies had shown that, regardless of the genuine role of CD6, it could associate with the T cell specific tyrosine kinases Lck and Fyn; we were therefore driven to explore further these associations using a novel method we had applied in the quantification of molecular associations at the T cell surface.

Based on our previous knowledge and the need to further understand the role of receptors implicated in immune responses, fundamental to explain cellular pathways, we proposed to study a group of structurally related members belonging to the Scavenger Receptor Cysteine-rich superfamily and their role in the immune system, and in T cells specifically. Thus, our specific goals were:

1 – To further characterize the Scavenger Receptor Cysteine-rich superfamily SRCR-SF, by a systematic analysis of the genome for new members

2 – To unveil the functional role of CD6 and evaluate its cytoplasmic associations with signaling effectors

3 – To assess the molecular organization and interactions of CD6 with the tyrosine kinases Lck and Fyn using Bioluminescence Resonance Energy Transfer (BRET)

As happens many times in the scientific process, some of our original ideas had to adapt to new developments in the field, and be altered as consequence of overlooked experimental complications. Firstly, the novel SSc5D protein that we have described has perhaps a more relevant role in innate immunity, as it is a secreted glycoprotein expressed mainly by monocytes/macrophages, and in epithelial cells of many organs, most notably in placenta; its role in T cell function may be proportionally less relevant. And although we have confirmed that CD6 has indeed a significant role in down-modulating T cell responses, contrarily to what was established, the analysis of its interactions with the kinases Lck and Fyn was not attained, as for still undetermined reasons we were unable to express the CD6 protein and mutants in the cellular system established for our BRET assays. Regardless of these difficulties, we proceeded with the analysis of the organization of the kinases and the features required for their function at the cell surface. Hopefully, the work presented in this thesis will contribute to a better knowledge of the immunological role of SRCR proteins, while it reinforces the idea that a clear understanding of a unifying role for SRCR proteins is still far away to be reached.

INTRODUCTION

1. The Immune System

"For every immunological stimulus or proinflammatory response, there is (are) one or more opposing control element(s)" (1)

1.1. Overview

Throughout evolution the immune system is possibly the one that has suffered more modifications and specifications. This fact is due to its extremely efficient components that altogether function as a very organized symphony. Single cell organisms started defending themselves by harnessing toxic peptides and genes that damage foreign molecules. These features are still present in more complex animals, and as multicellular systems evolved, specialized cells appeared having protein receptors that recognize pathogens, thus creating the first line of defense known as the innate immune system. In vertebrates, a second form of immunity appeared, the adaptive arm of the immune system. This second line of defense is maintained in the body as immunological memory. This "acquired" immunity emerged 450 million years ago in a fishlike creature and may be the result of DNA insertion from a virus or microbe (2, 3). Efficient adaptive immunity requires B lymphocytes, T lymphocytes and Natural Killer (NK) cells to protect against pathogens and cancer cells. Interactions between immunological players are needed for a proper immune response. These interactions can be directed with the usage of specialized receptors. These receptors are the T cell receptor (TCR), the B cell receptor (BCR), or other receptors such as cytotoxic lymphocyte-activating receptor (NKG2D), natural cytotoxic receptors or receptors of the signaling lymphocytic-activation molecule (SLAM) family, which have a horde of different combinatorial assemblies that can target a particular peptide and distinguish self from non self-antigens (Ags), avoiding the development of autoimmunity. All three lymphocyte types are derived from the same bone marrow hematopoietic stem cells. Although maturation of almost all hematopoietic lineages occurs in the bone marrow, T cell progenitors migrate into a specialized organ, the thymus, where they undergo thymic "education" and progress through multistage lineage commitment and differentiation to generate mature, self tolerant, functional T cells.

Activation of T lymphocytes involves the recognition by the TCR of a pathogen-derived peptide presented by the major histocompatibility complex (MHC) expressed on the surface of antigen-presenting cells (APCs), and co-stimulatory signals mediated by

additional T cell surface receptors. These events result in successive responses such as proliferation, migration, cytokine production and apoptosis. All these activating receptors that lead to lymphocyte activation are coupled to a variety of signaling cascades that are initiated through phosphorylation of tyrosine residues and the formation of complexes containing several adaptors and enzymes. Yet, a balance between positive and negative signals is required for T cell homeostasis. The main question regarding T cell activation is how the exogenous signal is transduced from the membrane to the nucleus and the precise sequence of events that takes place inside the cell.

This chapter will present an overview of the current knowledge and recent advances towards the understanding of basic events within the immune system from innate responses to the processes occurring at the T cell surface and the organization of its components.

1.2. The innate immune system

Despite the majority of the work presented in this thesis being mostly related to the adaptive immune system, it is appropriate to elaborate here on the events related to the innate immune system as this is the first and most basic immune response shared by almost all animals (and in a slightly different way by plants) since life emerged on earth. Although vertebrates are fortunate enough to have an adaptive immune system, we should consider that most of the organisms existing in the planet survive on innate immunity alone.

Before describing the main features of the innate immune system, it is important to refer that chemical and physical barriers are also included in the concept of a first line of defense. These are skin, mucociliary clearance mechanisms, low stomach pH, lysozyme in tears, saliva and other secretions. Innate immunity augments the protection offered by anatomical and physiological barriers (4). Cells that participate in this type of immunity include macrophages, neutrophils, dendritic cells (DCs), eosinophils, mast cells, NK cells and NKT cells from hematopoietic origin and epithelial cells from respiratory, gastrointestinal and genitourinary tracts, endothelial cells and fibroblasts that are non-hematopoietic. To improve these cellular defenses, innate immunity also has humoral components that comprise complement proteins, LPS binding protein (LBP), C-reactive protein and others.

The innate immune system senses the presence of microorganisms by germline-encoded pattern recognition receptors (PRRs). PRRs recognize conserved microbial

structures, known as pathogen-associated molecular patterns (PAMPs) that are essential for microorganism survival and difficult to change. They can also discriminate endogenous molecules released by damaged cells, called damage-associated molecular patterns (DAMPs). DAMPs can be a result of metabolic consequences of infection and inflammation (5). Different PRRs reacts with specific PAMPs which can divide them into four different classes: (a) Toll-like receptors (TLRs), responsible for sensing invading pathogens outside the cell and in intracellular endosomes and lysosomes (6); (b) C-type lectin receptors (CLRs), that recognize carbohydrates on microorganisms such as viruses, bacteria, and fungi in a calcium dependent-manner (7). CLRs either stimulate the production of proinflammatory cytokines or inhibit TLR-mediated immune complexes; (c) Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), which are normally modulated by ubiquitination and are able to trigger signaling cascades by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1; (d) NOD-like receptors (NLRs), a family of cytoplasmic proteins like RLRs that also recognize pathogens inducing transcriptional processing of pre-inflammatory cytokines and activate the inflammasome complex, nevertheless also regulate important non-inflammasome mechanisms (8). PRRs are not only expressed on macrophages and DCs but also in nonprofessional immune cells and they induce signaling pathways resulting in distinct anti-pathogen responses. Innate immune receptors detecting “missing self” molecules, expressed by normal and healthy cells, constitute another recognition strategy that triggers an inhibitory signal avoiding activation of an immune response against host tissues. This phenomenon is well exemplified by NK cells: the “missing self” concept was introduced to explain why NK cells target cells that have down regulated or no MHC class I at all on their surface (9).

Although sometimes innate immunity is simplistically separated from adaptive immunity, the latter has many built foundations on the innate processes. This can be exemplified by the enhanced capacity of neutrophils to kill bacteria when these are opsonized by antibodies produced through combined work of both T and B cells. Also, chemical defenses such as complement 3 (C3) can augment B cell effectors and memory function as demonstrated by cytotoxic T lymphocytes (CTL) that exhibit precise target specificity for their peptide antigens to the single amino acid level (10). Another representation of this immune interface is the critical role played by dendritic cells that function as APC, to empower T and B cell full activation. Additionally, regulatory T lymphocytes (T_{reg}) express TLRs and other immune receptors (11). Because innate and adaptive immunity have such synergetic efforts in maintaining homeostasis and survival, they evolved to be very efficient and redundant (12). Nonetheless, this equilibrium can be subverted by infection or disruption of tissue integrity (e.g. acute immunological pathology,

persistent infection, chronic inflammatory disease, autoimmunity and cancer) and it is crucial to completely comprehend the positive and negative pathways that regulate innate and adaptive resistance to modulate them clinically and to anticipate unfavorable effects of immunological therapies (13).

1.3. Scavenger receptor cysteine-rich proteins: at the cross-roads between the innate and adaptive systems

The scavenger receptor cysteine-rich (SRCR) superfamily was named after the structure found in the C-terminus of the type I scavenger receptor, a trimeric integral membrane protein found mainly on macrophages (14). SRCR domains are one of the few domains in which evolution allowed the development of a myriad of different proteins. They have a unique, stable and conserved structure, and versatility of these domains focused on slight changes in some amino acids that could freely evolve giving rise to an enormous functional diversity, whereas the pattern residues that compose the central core of the structure remained unchanged throughout evolution. Members of the SRCR superfamily are typically expressed on cells associated with the immune system (15). However, proteins containing SRCR domains were also shown to be synthesized by other cells such as epithelial cells and hepatocytes, and organs like kidney, placenta, stomach, brain and heart (16). Proteins containing SRCR domains can also have additional domains such as EGF, CUB, LCCL, or others. Functionally, the SRCR domains are thought to mediate protein-protein interactions and ligand binding thus mediating cellular differentiation and activation processes as shown in macrophages (17) and T cells (18).

On average, SRCR domains contain 100-110 amino acid residues displaying conserved well-defined spaced cysteine residues that form intra-domain disulphide bridges between them. Crystallography analysis revealed the structure of a SRCR domain with a central core formed by two antiparallel curved β-sheets cradling a α-helix (19, 20). The SRCR family is divided into two subgroups based on the organization of exons encoding each domain, the localization and number of cysteines. Group B SRCR members have 8 cysteine residues in each domain which is encoded by one single exon, whereas in group A, the domains have only 6 cysteines each and are coded by two exons. The SRCR domains have been well conserved throughout evolution (16).

Group B comprises the integral membrane lymphocyte differentiation antigens CD5 (21) and CD6 (22) and the structurally related soluble protein Spα, which, like CD5 and CD6, also contains three SRCR domains and is expressed exclusively by cells of

lymphoid origin (23). Many of the vertebrate SRCR proteins are implicated in development of the immune system and in the regulation of immune responses. To date, CD5 and CD6 are the most extensively studied SRCR members. Structurally, the two proteins are closely related, although the three extracellular SRCR domains are amongst the most divergent of the B group. Functionally, they have been assigned a role in mediating the binding of developing thymocytes with thymic epithelial cells and involved in modulation of signaling responses. Spα also has three extracellular SRCR domains, however it lacks a transmembrane domain (23). Spα is also known as apoptosis inhibitor expressed on macrophages (AIM), apoptosis inhibitor 6 (API6) or CD5-like molecule (CD5L), it can bind both myeloid and lymphoid cells an act as a promoter of macrophage survival.

Additionally, the group B SRCR family includes another six members of which CD163 (17) and M160 (24), both with a transmembrane region, were identified within the human monocytic lineage, and are regarded as a subgroup within group B molecules. CD163 has an important function that is the clearance of haemoglobin by binding haptoglobin and allows the formation on hapto-hemaglobin complexes, having a role in iron metabolism (25, 26). The soluble form can also participate in anti-inflammation, which confers to CD163 a cytokine-like function. Recently, CD163 was shown to be a new potential scavenger receptor of TNF-like inducer of apoptosis (TWEAK) which confers a function in atherosclerosis (27). DMBT1 is the largest member of the family as it comprises 14 SRCR-encoding exons spaced by SRCR-interspacing domains (28). It was described based on a deletion in a medulloblastoma cell line. DMBT1 can be secreted or associated with the plasma membrane of macrophages, although the presence of a transmembrane domain was not characterized yet. Once in the membrane, DMBT1 can bind surfactant protein D (SP-D), which acts as an opsonin to facilitate the removal of pathogens through interactions of its C-type lectin domain with carbohydrate and glycolipid structures on the surface of a wide range of microbial targets, such as viruses, bacteria, yeasts and fungi (29-31).

The most recent members of the family are S4D-SRCRB (32), SSc5D (33) and SCART (34). S4D-SRCRB and SSc5D have four and five group B feature domains, respectively. SCART has eight putative SRCR domains encoded in the genome but Holm and colleagues have only identified five expressed SRCR domains. Both SSc5D and SCART show to have alternatively spliced forms resulting in smaller proteins, however little is known about their function or binding properties. Definition of this subgroup lies on the exclusive presence of SRCR domains in their extracellular regions; however, no function has yet been assigned.

Regarding this study, it is important to decipher the main features of the two most well known members of this SRCR-SF group B, CD5 and CD6.

1.3.1 The glycoprotein CD5

CD5 was one of the first T cell surface glycoproteins to be identified. Curiously, it comprised three SRCR domains in its extracellular region instead of the common Ig-like domains described for most others T cell markers at the time. CD5 is also expressed in thymocytes and in a subset of B cells, B1a, and on malignant chronic lymphocytic leukemia (CLL) B cells (35, 36). Variation of CD5 expression levels by T or B cells can setup thresholds for different cellular responses (37). For instance, in B cells the transcription usage of an alternate exon 1 can induce CD5 to be expressed at the cell surface or being retained intracellularly with its expression varying inversely between each isoform (38). Dowregulation of the cell-expressed isoform leads to a decrease in the threshold of BCR signaling. Interestingly, in B-CLL this form is the most predominant and is constitutively tyrosine phosphorylated suggesting a chronic stimulation through the BCR and not through CD5 resulting in a prosurvival mechanism (39).

CD5 is a 67-kDa protein composed of a highly conserved cytoplasmic domain including Thr/Ser and Tyr phosphorylation sites and containing two potential immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (21). Recently, it was suggested that CD5 can have homophilic interactions through its SRCR-domain 1 (d1) (40); however, CD5 was also reported to bind to different molecules expressed by APC, such as CD72 (41), gp40-80 (42), gp150 (43), and IgV(H) framework region sequences (44). Although several studies tend to demonstrate that a physiological ligand for CD5 exists, any definitive interactions have not still been confirmed, making this an open field to investigation. CD5 has also been described to bind in cis to other proteins like CD2 (45, 46) and the BCR, through its d2 (44).

Initially, CD5 was regarded as a co-stimulator of T cells although this observation was mainly based on studies that used antibody-crosslinking stimulations and thus enhancing T cell proliferation (47, 48). But when the CD5 knockout mouse was constructed, a clear inhibitory role on TCR signaling was assigned for CD5 (18). The exact manner on how such an inhibitory signaling is achieved is not known. Nevertheless, several studies demonstrated CD5 interactions with different negative regulators, including SHP-1, Ras-GAP, c-Cbl and CK2 (49-51), that could explain the inhibitory properties of CD5. The cytoplasmic domain of CD5 is rapidly phosphorylated upon

TCR/CD3 stimulation (52). The kinase responsible for this phosphorylation is probably Lck, although both Fyn and Itk may complement or regulate its function in this context (53-55). Moreover, besides being present at the immunological synapse upon activation, CD5 exerts an inhibitory effect on it possibly via its ITIM sequences. Such effect is increased when there is an upregulation of CD5 levels at T cell surface (56). CD5 can also regulate T and B-1a cell survival as demonstrated by an attenuated experimental autoimmune encephalomyelitis (EAE) in $CD5^{-/-}$ mice (57). Another study evidenced an inverse correlation between CD5 expression levels on human tumor antigen-specific CD8 CTLs and susceptibility to AICD, where CD5 promoted the survival of CTLs through the downregulation of FasL and thus inhibiting caspase 8 activity (58).

1.3.2 The glycoprotein CD6

CD6 is a type I membrane glycoprotein and, as mentioned earlier, it contains three extracellular SRCR domains (22). It is expressed at low levels on immature thymocytes and at high levels on mature thymocytes. During thymic maturation, the expression of CD6 is tightly regulated, contributing for thymocyte selection. Immature double negative $CD4^{-}CD8^{-}$ and double positive $CD4^{+}CD8^{+}$ thymocytes express CD6 but the higher expression is observed at single positive stages (59). The majority of peripheral blood T cells, a subset of mature B cells and some B cells derived from lymphocytic chronic leukemia (B-CLL), and a subset of neuronal cells also express CD6.

The *CD6* gene is located on chromosome 11, in the region 11q13.1, contiguous to the *CD5* gene; both have probably arisen from an ancestral gene, which explains their similarity in domain organization and pattern of cellular expression (60). In mice, *Cd6* and *Cd5* genes are located on chromosome 19. The *CD6* promoter has been recently described and the transcription factors RUNX1/3 and Ets-1 are important regulators that control CD6 expression in T lymphocytes (61). CD6 is a 668 amino acid-long protein with a 24 aa predicted signal sequence, a 374 aa extracellular domain, a 23 aa transmembrane region and a 244 aa cytoplasmic tail. The 626 amino acid murine homolog has also been identified (54). The human and murine proteins share 70% identity at the protein level. It was demonstrated that a single-nucleotide polymorphism (SNP) in the *CD6* exon 1 is associated with susceptibility to multiple sclerosis (MS) (62), and is also correlated with a lower expression of the molecule. Other SNPs were also identified but their effects remain elusive (63). Apart from MS, In recent times CD6 has been linked to other autoimmune diseases, including rheumatoid arthritis (RA) (62, 64,

65). Several different isoforms of CD6 can occur after alternative splicing of SRCR domains (66) or cytoplasmic domain-encoded sequences (60).

The molecular mass of the protein can vary from 105 kDa to 130 kDa; this difference in size results from the heavy glycosylation that can occur within SRCR molecules. CD6 has eight putative *N*-glycosylation and two putative *O*-glycosylation sites on its extracellular part. The large cytoplasmic tail of CD6, despite the absence of intrinsic catalytic activity, has several amino acids that could be targets for important signaling molecules; those are 9 tyrosine, 33 serine and 12 threonine residues. It also has two putative proline-rich sequences (PxxP where x denominates any amino acid residue) which could be docking sites for SH3 containing proteins. However, the signaling pathways have not been elucidated. Two studies have demonstrated the binding of SLP-76 and syntenin-1 to human CD6 tyrosine (Y) 662 (67, 68). SLP-76 is known to be phosphorylated by ZAP-70 (69) and interacts with Grb2 and PLC-γ linking the protein with Ras and calcium pathways in T cells. Also ADAP and HPK1 bind to the SH2 domain of SLP-76, but for CD6 binding, Y662 needs to be phosphorylated. Syntenin-1 is another adaptor protein able to bind cytoskeletal proteins and signaling effectors. In rat, CD6 can associate with different tyrosine kinases, such as Lck, Fyn, Itk and ZAP-70 (54). In T and B cells, phosphorylation of CD6 tyrosine residues resulted from the activation with PMA, anti-CD3 or CD2+CD4, inducing the interaction with kinases (70, 71). Cross-linking of a CD6 mAb resulted in activation of the Erk1/2, JNK and p38 kinases, thus showing the involvement in the MAPK cascade (72). CD6 has also been described to have a mitogenic effect for T cells, shown with some CD6 specific monoclonal antibodies in conjunction with either accessory cells or PMA and anti-CD2 mAb, supporting the concept of CD6 as a co-stimulatory molecule (73). Other studies characterized CD6 as an adhesion molecule within thymocyte-thymic epithelial cell interactions (74). Our group has recently reported a different role for CD6 suggesting that, like CD5, CD6 is a negative modulator of T cell activation as it can inhibit calcium responses and T cell proliferation after T cell activation both super Ag (sAg) or mAb-triggered activation of the TCR/CD3 complex, and that this inhibitory effect is mediated by its cytoplasmic tail (75).

The physiological counter receptor of CD6 is activated leukocyte cell adhesion molecule (ALCAM or CD166), a cell surface receptor belonging to the Ig superfamily (IgSF), having 5 extracellular Ig-like domains (74, 76). CD166 is widely expressed in several tissues and cell types, including bone marrow stromal cells, thymic epithelial cells, activated T and B cells, denditric cells, neurons, fibroblasts, endothelial cells, and keratinocytes (77). Besides being the ligand for CD6, CD166 can also mediate homophilic

interactions (78). Binding studies have shown that membrane proximal SRCR domain (d3) of CD6 and the N-terminal V-like Ig domain (d1) of ALCAM are sufficient to mediate the interaction (78, 79). The CD6-CD166 interaction has provided the first description of an SRCR-ligand association, and although this type of binding cannot be generalized to all SRCR interactions, it established a framework to study the structure and function of other SRCR proteins. Binding of CD6 to CD166 can stabilize the interaction between a T cell and an APC. Blocking this interaction can reduce T cell-APC contacts and the localization of CD6 in the immunological synapse is abrogated when T cells express an alternative spliced isoform, CD6Δd3, devoid of the ligand-binding domain (66). CD6-CD166 interactions have been postulated to play a role in thymocyte development (59).

Other ligands were also assigned to CD6 based on the observation that recombinant CD6 precipitates two other proteins (besides CD166) from human epithelial cells, with 45 and 90 kDa (80-82). These possible interactions were suggested to be mediated by CD6-d1 or d2 and induce different, still unidentified, functions for CD6. Recently CD6 was reported to bind to PAMPs and LPS resulting in the activation of the MAPK signaling pathway (83).

At the plasma membrane level, the association in *cis* of CD6 with CD5 is the only described between SRCR proteins. CD5 and CD6 can physically associate as shown by confocal microscopy coupled with FRET, and after T cell activation both proteins are expressed at immunological synapses and colocalize with the TCR/CD3 complex (84). Another study showed that CD6 is able to physically interact with the TCR/CD3 complex while being part of central supramolecular activation clusters (cSMACs) of the immunological synapse, participating in the induction of T cell proliferation (85). The CD5-CD6 association occurs through extracellular domains given that both wild-type CD5 and a CD5 form lacking the cytoplasmic tail are able to co-precipitate CD6. Interestingly, when CD6 and CD5 are physically associated they are both hyperphosphorylated at Y469 and Y429, respectively. This may suggest that this interaction can contribute to TCR or BCR-independent activation (54).

1.4. Interface between innate and adaptive immune system

One of the main goals of this thesis is to characterize the function of SRCR proteins, which can enormously diverge in their expression as well as in their function. In fact the title of this work clearly shows that this family of proteins has a significant role between

innate and adaptive immune responses. Such interface can be explored in the different expression of SRCR-containing proteins in both macrophages and T cells.

The innate immune system, as mentioned before, has macrophages and neutrophils to provide the first line of defense against microorganisms and bacterial infections. However, such groups of cells cannot always recognize and eliminate infectious pathogens. As this happens, another immune response approach evolved to provide a more adaptable way of defense increasing the protection of the individual. This protection is given essentially by lymphocytes. The processes that take part in the achievement of the adaptive immune response are given in the following sections.

1.5. The adaptive immune system

The great efficiency of vertebrates in somatic diversification processes gave them an astonishing advantage to survive in an environment full of pathogens. They can generate a repertoire of structurally diverse antigen receptors expressed mainly by two different lineages of lymphocytes: B cell that produce antibodies and T cells that can have cytolytic and helper functions (86). The development of both lineages is finely controlled by the coordinated action of transcription factors (87); these lineages can also be divided in sublineages for both B cells, such as B1 and B2 cells (88), and T cells also with two main sublineages, one expressing an αβ T cell receptor (TCR) and the other expressing a γδ TCR (89). T and B cells develop at separate anatomical sites, thymus for T cells and the latter in the bone marrow or fetal liver.

Adaptive antigen receptors expressed at the membrane or soluble forms have distinct functional characteristics compared with innate receptors: they suffer clonal expression, alloreactivity and can interact with the aid of chemokines and/or cytokines and generate specific memory responses. The next section will deepen the concepts that explain the adaptive immune response, especially those related to T cell responses.

2. T lymphocytes and signaling

2.1. TCR lineage

T cell receptors recognize foreign and self peptides presented in the context of the major-histocompatibility complex (MHC) (90). The TCR/CD3 complex is a multimeric transmembrane complex consisting of variable disulphide-linked αβ chains (or γδ in a sub-

set of T lymphocytes) associated with non-polymorfic CD3 proteins. CD3 oligopeptides comprise CD3γ, CD3δ, CD3ε, and TCRζ chains. The TCRα and TCRβ chains (or TCRγ and TCRδ) each contain a clonotypic variable (V) immunoglobulin (Ig)-like domain which binds to peptide-MHC (pMHC) complexes on the surface of APCs or target cells; each chain also has a constant (C) Ig-like domain. The variable domains display three complementarity determining region loops (CDR) generated by the rearrangement of, in the case of TCRβ or TCRδ, segments known as variable (V), diversity (D) and joining (J) elements. TCRα and TCRγ contain only V and J segments. The fusion of these elements at a DNA level results in the formation of a functional VDJ gene unit (or VJ in the case of TCRα or TCRγ) encoding the variable domain (91). The combinatorial association of V(D)J elements enforces the larger diversity in the immune system.

The TCR/CD3 complex is unique as it comprises 10 immune receptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the complex. CD3γ, CD3δ and CD3ε chains have each a single ITAM, whereas CD3ζ chains possess 3 ITAMs each. These cytoplasmic motifs are mostly responsible for signal transduction. The possible role for such a high number of ITAMs could be to recruit different signaling molecules that possibly induce separate activation pathways. The TCR/CD3 complex is distributed into TCRαβ, CD3γε and CD3δε heterodimers and a TCRζζ homodimer (92). Several studies established that the intracellular domains of CD3ε and CD3ζ but not CD3δ and CD3γ associate with the acidic inner leaf of the membrane (93-98). However Kuhns and colleagues proposed a model where the two CD3ε intracellular subunits emerge from the membrane side-by-side and interact with the acidic inner leaf of the membrane. The CD3δ and CD3γ domains emerge from the membrane to flank CD3ε on the one side of the TCR and CD3ζζ would localize and associate with the acidic inner leaf membrane below the unique surface of the Cα domain on the other side of the TCR (99). Thus, the four ITAMs of CD3δε and CD3γε and the six of CD3ζζ would be in opposite sides of the TCR (100).

As mentioned earlier, T cells can be divided into two large classes: αβ and γδ T cells. The first choice that progenitor cells experience when they commit to a T cell-lineage is the decision to become αβ or γδ T cells. These lineages refer to the TCR type. Three out of four TCR loci are rearranged at the double negative (DN) ($CD4^-$ $CD8^-$) thymocyte stage: *TCRB, TCRG* and *TCRD*. If the cell succeeds to an in-frame *TCRB* rearrangement it will express TCRβ in complex with the germeline-encoded pre-TCRα, giving rise to a burst of proliferation and upregulation of the CD4 and CD8 coreceptors, *TCRG* silencing and *TCRA* rearrangement initiation. Double positive (DP) cells ($CD4^+CD8^+$) will express TCRαβ on their surface and can further differentiate towards

single $CD4^+$ or $CD8^+$ lineages (101). The DP stage is possibly the hallmark for αβ lineage commitment. So if there is a lack in DP progression, the γδ lineage arises and becomes functionally mature (102). Even though not in a deterministic way, the TCR can influence lineage decision as shown in wild type (wt) mice where the majority of $TCR\gamma\delta^+$ precursors choose the γδ, and pre-TCR expressing precursors the αβ lineage (103). Nevertheless two studies suggested that TCR signal strength instead of TCR class can determine lineage choice (104, 105).

2.2. TCR-pMHC interaction

The first structure of a TCR was published in 1996 describing a murine TCR (2C) binding to dEV8 peptide associated with MHC class I H2-K^b (106). In the same year the human TCR (A6) structure complexed with HLA-A*0201-Tax (peptide derived from human T cell lymphotropic virus type 1) was also determined (107). These structures provided the first insights into T cell antigen recognition and demonstrated several characteristic of the interface between the TCR and pMHC. Currently, there are about 25 human TCR/pMHC complexes solved, which seems relatively few compared with the reported number of antibody and non-ligated pMHC structures. This lack of structural information compromised the formulation of a comprehensive method to explain T cell antigen recognition. The way TCRs interact with pMHCs is relatively conserved as TCRα chains bind diagonally to the MHC α2 domain and the TCRβ chain contacts the MHC α1 domain. This interaction occurs between the pMHC surface and TCR complementarity determining region loops (CDR) (108). These CDR-loops have different functions upon the TCR-pMHC interaction: variable (V)-gene encoded CDR2-loops contact the helical region of the MHC-surface, the V-gene encoded CDR1-loops can contact both MHC and peptide and the more variable somatically rearranged CDR3-loops contact mainly the antigenic peptide (109). For TCR triggering to occur, it just needs a single agonist peptide-MHC ligand (110-112).

The central role of the TCR-CD3 complex in immunity is a prime motivator for understanding the molecular mechanisms associated with its function. Such knowledge will give practical clues to develop T cell responses in vaccines, or towards tumors or pathogens. Although the assembly of the TCR-CD3 complex does not allow for the incorporation of exogenous TCR on cells expressing endogenous peptides, it is important to better understand how the complex assembles and functions, for a consistent guidance towards immunotherapy (113). Definitely, more insights into how all pieces of this

molecular machine fit and work together are needed to fully characterize one of the most fundamental questions in immunology.

2.3. TCR signaling

T cell responses to agonistic MHC-ligands result in the activation of a network of signaling molecules, second messengers and accessory proteins to transmit information. The initial TCR interaction with the ligand starts within 0.25 s, once the T cell surface makes contact with the APC and about 1 s after the first biochemical changes happen - phosphorylation of CD3ζ subunits in the TCR complex which initiates the T cell signaling cascade. At first, CD3ζ ITAMs are phosphorylated by Lck and bound by another protein tyrosine kinase, ZAP-70. Double-tyrosine residues within these ITAMs are phosphorylated, becoming docking sites for the tandem SH2 domains of ZAP-70. The catalytic activity of ZAP-70 is essential for conventional T cells but not for regulatory T cells (114). The exact mechanism by which TCR-pMHC interactions initiate net phosphorylation is still unclear, but the explanation could be the simple local exclusion of phosphatases, such as CD45 (115).

This first phase of T cell activation is co-receptor independent. After ZAP-70 binds to CD3, the co-receptors CD4 or CD8, associated with the Src family kinase Lck, are recruited and associate with TCR-CD3-pMHC complex. This association stabilizes TCR-pMHC interactions and favors continuous phosphorylation by Lck of CD3 elements, ZAP-70 and other downstream targets. A recent biophysical study showed that the initial contact of TCR to pMHC induces, in a Src-family kinase dependent manner, the binding of CD8 to pMHC (116). Another study demonstrates that high density of self pMHC promotes their interaction with CD8 playing a critical role in the efficient recognition of the TCR with a non-self peptide (117). Several lines of investigation demonstrate that phosphorylation of CD3 occurs through Lck (or Fyn) that is not bound by the co-receptor; however the mechanism that starts this signaling cascade is defectively characterized. It is clear that co-receptors are not absolutely required for signaling as some T cells are activated in a co-receptor independent manner (118, 119). Other evidence is that thymocytes need Lck to develop but the presence of CD4 or CD8 is not compulsory (120, 121). A highly conserved motif within the TCR – TCRα-chain connecting motif (α-CPM) is important for CD8 association enhancing T cell responses (122, 123) and it also contributes for T cell activation and positive selection (124). The α-CPM region is believed

to interact with the membrane proximal part of CD8β in a “co-receptor zipper” mechanism, enabling Lck to utterly phosphorylate CD3 ITAMs (125).

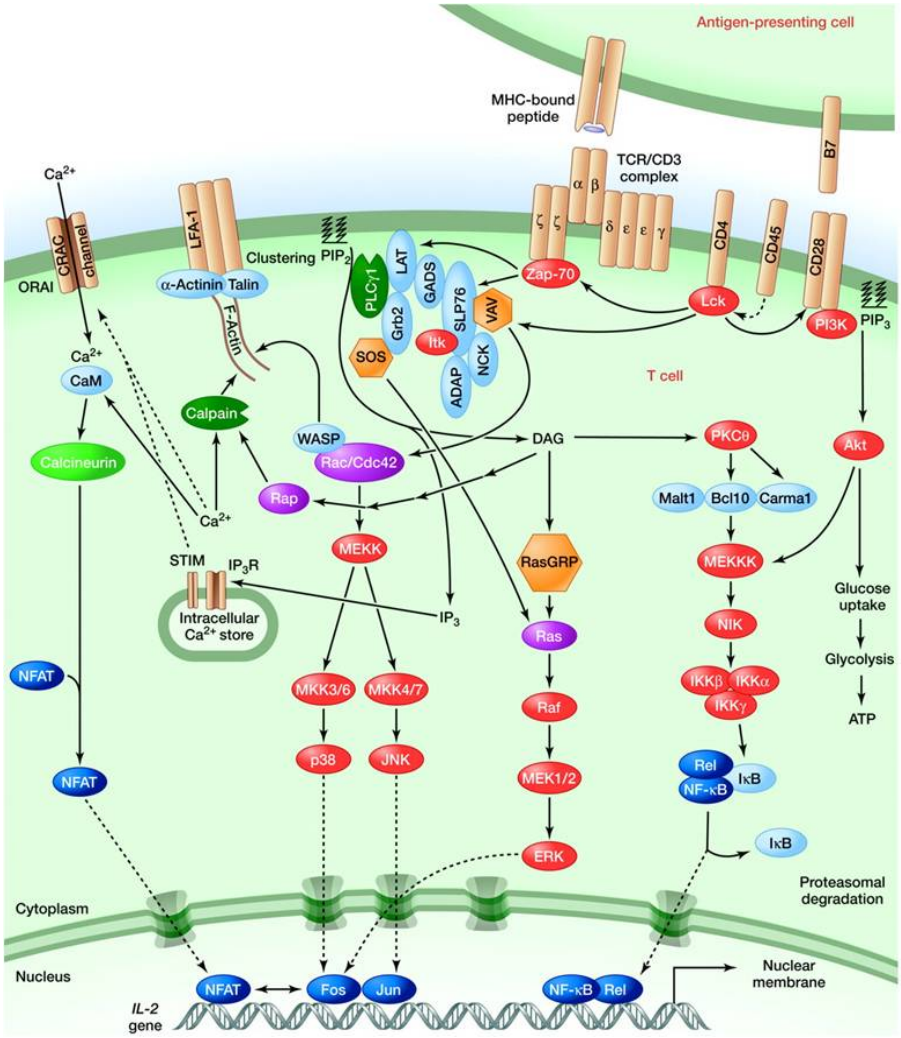

Figure 1 – **T cell receptor signaling**. Signaling starts with engagement of the TCR to pMHC. The TCR/CD3 complex also comprises the non-polymorphic CD3 γ, δ, ε and TCRζ subunits. Co-receptors, CD4 in helper T cells and CD8 in cytotoxic T cells, are also important. Lck and ZAP-70, members of the Src and Syk kinase families, respectively, are critical signaling molecules closely associated with the receptors and co-receptors. Activation of tyrosine kinases is the first biochemical change that follows receptor engagement. These activated kinases phosphorylate several adapter proteins and signaling enzymes, e.g adapters LAT and SLP-76, and induce the assembly of multiprotein complexes that include enzymes such as phospholipase Cγ1 and Vav. Activation of an additional protein tyrosine kinase, Itk, occurs within these complexes. These events occur at the plasma membrane, where critical lipid substrates of enzymes such as PLCγ1 and PI3 kinase (PI3K) are located (Image taken from Samelson (126)).

The second stage in TCR signaling is the phosphorylation of LAT and SLP-76 by ZAP-70. As a transmembrane adapter protein, LAT joins upstream signaling of Lck/ZAP-70 to downstream signal events that include calcium mobilization, phosphatidylinositol turnover and Ras activation. Activation of LAT and SLP-76 recruits to the TCR vicinity

tyrosine kinases that phosphorylate phospholipase Cγ1 (PLCγ1) and Vav1 (127, 128). Also, Itk activation occurs within these complexes (129). All events take place in the plasma membrane where PLCγ1 and PI3 kinase (PI3K) are located, both catalyze the recruitment of second messengers such as inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) that trigger Ca^{2+} flux and contribute to protein kinase C (PKC) and Ras activation.

2.4. Models of TCR triggering

How does the T cell receptor signaling begin? Extensive research conducted to answer this question has been made throughout the years. Despite many attempts to address this question, there is still no consensus regarding a definitive explanation. It is well established that T cell activation begins when the TCR binds to peptides presented by MHC molecules at the APC membrane. But how the signal is transmitted to the cytoplasmic subunits of the TCR/CD3 complex is still controversial. Several models have been proposed, based on essentially TCR aggregation, conformational changes, and segregation or redistribution of the complex (130).

Some studies have demonstrated that T cells activated by APC or soluble pMHC could induce TCR aggregation (131-133). This TCR aggregation leads to the formation of “microclusters”, which were first visualized by total internal reflection fluorescence (TIRF) microscopy of T cells in a lipid bilayer (134, 135). In addition, some evidence showed that pre-formed TCR aggregates already exist at cell surface (136-139). The co-receptor heterodimerization model postulates that the CD4 or CD8 co-receptors bind to the same antigen-MHC complex bringing Lck closer to the CD3 cytoplasmic ITAMs in order to promote their phosphorylation (140). In this way, clustering of TCRs by antigen recognition helped by the interaction of another TCR with self-peptide and the recruitment of co-receptors bound to Lck would initiate the signaling cascade (141, 142). Other models regarding conformational changes have been proposed, the appeal of these models is that TCR can be triggered at very low-levels of agonist peptide-MHC molecules. Conformational changes in CD3 intracellular domains can allow adaptor protein interactions (143) and accessibility of ITAMs for phosphorylation (96). Recently, a model addressed a piston-like movement of the CD3 cytoplasmic tail relative to the plasma membrane. This model supports a mechanical pulling of the TCR-pMHC interaction and a torque on the CD3 subunits driving to signal transduction (144, 145). A third type of mechanism that can explain TCR triggering is segregation or redistribution of the TCR-

CD3 complex towards other membrane-associated molecules. When the TCR-pMHC interaction takes place, larger and bulkier protein tyrosine phosphatases (PTPs) like CD45 should be pushed away to the periphery of the immunological synapse. Transference of inhibitory regulators could thus aid phosphorylation, as a result of enrichment of these areas with Src family kinases and signal transduction to occur (146). All these models are not necessarily mutually exclusive but rather elements of each of them can cooperate resulting in T cell activation (147).

3. Receptors implicated in TCR signaling

Besides conventional pathways implied in T cell signaling, it is well established that other effective co-stimulatory and co-inhibitory pathways involving interactions of proteins have important roles in immune responses. In particular, when T cell activation via CD28, the first described co-stimulatory receptor, is impaired. A number of receptors expressed on T cells have been characterized to act as co-stimulators or co-inhibitors upon ligation with their counter-receptors expressed on APCs; however, some of these effects were based only on their ability to generate second signals when crosslinked with antibodies. Thus, the biochemistry and biology of other receptors remains much less understood than that of the TCR. Therefore, the next section describes the characterization of different receptors implicated in T cell activation and signaling and their physiological roles.

3.1. The coreceptors CD4 and CD8

During thymic maturation, developing thymocytes adopt two possible lineages defined by the expression of the co-receptors CD4 or CD8, and have specific functions and antigen affinities. Each lineage originates two types of lymphocytes, the CD4 helper T cells restricted for MHC-II, and CD8 cytotoxic T cells (CTLs) that bind MHC-I (148, 149). CD4 and CD8 act as auxiliary binders that tighten the interaction between the TCR and pMHC; moreover, they also have key roles in signal transduction mechanisms.

Both proteins are structurally related, CD4 has four Ig-like domains in the extracellular domain and CD8 has one, followed by a transmembrane domain and a short cytoplasmic tail. One major difference is that CD4 is normally expressed at the membrane as a monomer, although some studies suggest that it could dimerize through the Ig-d4 (150), while CD8 can be expressed as a disulphide-linked heterodimer (αβ) or homodimer

(αα). CD8 (αβ) is more effective as a co-receptor suggesting that it is the primary to bind MHC-I (151). It can also be palmitoylated, resulting in its localization in lipid rafts (152), like Lck that was also demonstrated to be targeted to rafts through palmitoylation and myristoylation; therefore the presence of both Lck and CD8αβ in lipid rafts provides a motif and an opportunity for their interaction, explaining why CD8αβ functions as a stronger co-receptor (153). The homodimer CD8αα has been described in a subset of regulatory T cell populations (154) and indeed it might be a negative regulator of T cell activation (155).

Recently, the crystallization of a TCR-pMHC-CD4 ternary complex showed an arch in which both TCR and CD4 are tilted instead of being oriented vertically. The apex of the arch is formed by the α2 and β2 domains of HLA-DR4 and the d1 domain of CD4. The TCR makes an angle of ∼60° with the T-cell surface and the CD4 molecule an angle of ∼70°; the apical angle between pMHC and CD4 is ∼50° (156). Regarding this structure, CD4 dimerization cannot physically occur as CD4 is monomeric in the TCR-pMHC-CD4 crystal. This model does not consider the possible binding of CD4 to the TCR-CD3 complex, as the CD3 complex is absent. CD4 or CD8 are also geometrically competent to deliver a maturation signal to double-positive thymocytes during T cell selection.

3.2. The B7-CD28 superfamily

The B7-CD28 superfamily consists of the following receptor/ligand pairs: CD28 and cytotoxic T lymphocyte antigen 4 (CTLA-4) that share the same ligands CD80 and CD86; inducible T cell co-stimulator (ICOS) with LICOS; and programmed death 1 (PD-1) with both PD-L1 and PD-L2. Other members such as B7-H3 and B7-H4 have no identified ligands. This family is involved in T cell co-stimulatory and co-inhibitory processes.

CD80 and CD86 have particular characteristics as they bind the stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (157). CD28 can be regarded as one of the most potent co-stimulatory molecules after TCR antigen recognition, as it augments and sustains T cell responses promoting T cell survival and enabling the release of cytokines to initiate T cell differentiation and clonal expansion (158). CTLA-4 engagement delivers negative signals to the TCR, counterbalancing the positive stimulus given by CD28 and inhibiting cytokine release and cell cycle progression (159). Structurally, both molecules have a transmembrane domain and have a single variable Ig-like domain (160). CD28 and CTLA-4 are both dimers, but they diverge in their ability to bind ligands. CTLA-4 interacts with CD80 and CD86 with higher affinity and avidity than does CD28, with CTLA-4-CD80

being the strongest and CD28-CD80 the weakest (161). Although these interactions have low affinity compared with other molecular interactions, they have very fast kinetics probably to enhance T cell scanning on APC surfaces (162). Constitutive expression of CD28 on the plasma membrane of resting and activated T cells is in opposition to the expression of CTLA-4 only in activated cells (163). In contrast to conventional T cells, CTLA-4 is always expressed on T regulatory cells (T_{reg}) (164) conferring a crucial role in regulating peripheral T-cell tolerance. Although coordinated signals through CTLA-4 and CD28 are still not clear, probably CTLA-4 inhibits T cell responses by out-competing with CD28 for binding to B7 molecules inducing immunosuppressive cytokines or by directly antagonizing CD28 signals and/or TCR-mediated signaling. It was also shown that CD80 can bind to PD-L1 and this interaction can downregulate murine T cell responses (165).

Another member of this family is the membrane receptor PD-1 (or CD279). It is composed of one Ig-like domain and a cytoplasmic domain containing two tyrosine-based signaling motifs: one ITIM and one immunoreceptor tyrosine-based switch motif (ITSM). Similar to CTLA-4, the interaction of PD-1 with its ligands PD-L1 and PD-L2 delivers co-inhibitory signals. Phosphorylation of a tyrosine residue within the ITSM motif promotes the recruitment of SH2-domain containing tyrosine phosphatase 2 (SHP-2), and possibly SHP-1, to the cytoplasmic tail of PD-1, which will down-regulate the CD28-mediated PI3K activity, thus decreasing Akt activation (166). PD-1 can also inhibit other pathways as it prevents phosphorylation of CD3, ZAP-70 and PKC (166). But in contrast with CTLA-4 and CD28 dimerization, PD-1 exists as a monomer because it lacks the membrane proximal cysteines required for homodimerization (167). PD-1 is expressed on peripheral $CD4^+$ and $CD8^+$ T cells, B cells and monocytes upon activation. Expression of PD-1 is in part mediated by the recruitment of nuclear factor of activated T cell c1 (NFATc1) to the nucleus (168). PD-1 ligands have Ig-V-like and Ig-C-like extracellular domains and a short intracellular domain. They have distinct patterns of expression: PD-L1 is constitutively expressed in myeloid and lymphoid cells and is up-regulated after activation, whereas PD-L2 expression is restricted to macrophages and DCs in response to cytokines (169). PD-1 has an additional tolerance mechanism by promoting T_{reg} development and function (170).

ICOS is expressed upon T cell activation and binds LICOS (171). They are both transmembrane proteins belonging to the Ig superfamily. ICOS is a disulphide-linked homodimer with extracellular IgV domains having 24 and 17% amino acid (aa) homology with CD28 and CTLA-4, respectively (172). ICOS ligation promotes T cell proliferation and the production of diverse effector cytokines such as IFN-γ, IL-4 and IL-10. However, unlike CD28, ICOS minimally induces IL-2 production, suggesting that its interaction triggers a

positive co-stimulatory signaling cascade different from the one given by the CD28-CD80/86 interaction (173). In agreement with a positive role in T cell stimulation, ICOS-deficient mice exhibit severely affected T cell activation and proliferation alongside with a reduction in T and B cell responses, an insufficiency in Ig class switching and impaired germinal center formation (174).

The orphan ligand receptors named B7-H3 (CD276) and B7-H4 are type I membrane proteins with IgV-IgC domains. Both mRNA are widely express on human tissues, but protein expression is relatively rare suggesting that a tight post-transcriptional control is a key regulatory mechanism of its expression (175, 176). B7-H3 was first shown to be a co-stimulatory molecule (175) but other studies suggested an inhibitory function for this receptor (177). B7-H4 is a co-inhibitor of T cell responses via repressing $CD4^+$ and $CD8^+$ T-cell proliferation, cytokine production, and generation of alloreactive CTLs, by arresting the cell cycle (176). These molecules play critical roles in immune responses outside the adaptive immune system. They are both broadly expressed in many tissues and cells and found to regulate host innate responses by suppressing growth of neutrophil progenitors (178) and regulate osteopoiesis in addition to T-cell responses (179).

3.3. The accessory receptor CD2

CD2, alongside the B7 and CD28 families, is part of the large Ig superfamily. The interactions of CD2 with CD58 (a glycosylphosphatidylinositol GPI-anchored protein on APC) in humans (180), or with CD48 in rodents, resulted in the first co-stimulatory signaling pathways identified on T cells (181). CD2 is a 45-58 kDa type I transmembrane protein expressed on all T cell lineages and NK cells. It comprises on the extracellular part two IgSF domains, of which the membrane-distal V-like domain is involved in the binding to the ligand (182). CD48 and CD58 have a similar structure to CD2, displaying two Ig-like domains and a short stalk, with the N-terminal region mediating the interaction. CD2 associations were shown to be at low affinity but with high avidity (183). The CD2-CD58 interaction is important as it stabilizes T cell-APC interaction in a low antigen environment by maintaining the cell-cell contact.

In addition to function as an adhesion molecule CD2 can also have an effect on signaling. It has been described that the CD2-CD58 interaction induces signaling cascades with the involvement of Lck, TCRζ chain, and LAT clustering through actin-mediated recruitment into membrane microdomains (184). Moreover, cross-linking of CD2 can induce T cell proliferation and cytokine production in a ZAP-70 dependent manner

(185). On the other hand, CD2 can also bind to the inhibitory receptor CD5 modulating signals at the T cell surface (45, 46). Interaction of CD2 with the Src family tyrosine kinases Lck and Fyn was also demonstrated by CD2 cross-linking leading to an increase in Lck kinase activity (186). This physical interaction is mediated by the cytoplasmic domain of CD2 and occurs in specific domains within lipid rafts (187, 188). This interplay is probably mediated by the kinase's SH3-domains binding to proline-rich sequences of the cytoplasmic domain of CD2 (189, 190). In addition, CD2 binding to CD58 on APC activates PLC-γ and augments intracellular calcium release in antigen-specific T cell clones (191). For all these facts it is possible to assert that the CD2-CD58 interaction in human T cells displays the potential to initiate TCR signaling independently and/or synergistically with TCR stimulation. In NK cells, CD2 can have two distinct functions: as a co-stimulatory receptor that regulates the development of NK effector functions and as an activating receptor towards CD58 expressed on target cells to promote their lysis (192).

4. Machinery involved in TCR signal transduction

4.1. The role of adapter proteins

Central to the process of immunoreceptor signaling are the adapter molecules, they can propagate and diversify the immunoreceptor-initiated signal by triggering immune effector functions. Traditional adapters are composed of protein-protein or protein-lipid interaction domains and motifs. Examples are the growth factor receptor bound protein 2 (Grb2), linker of activated T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), signaling lymphocytic activation molecular (SLAM)-associated protein (SAP) and ITAM-subunits. However motifs present in adapter proteins can also be found in molecules with intrinsic catalytic activity, like PLCγ and the ubiquitin ligase c-Cbl, which can intervene with true adapter-like functions and modulate the formation of signaling complexes.

4.2. Kinases and phosphatases involved in T cell signaling

Activation and maintenance of T lymphocytes involve a complex network of signaling events in which tyrosine phosphorylation and dephosphorylation play a critical role. As mentioned earlier, the first major signaling event taking place after engagement of the TCR with pMHC is the activation of tyrosine kinases, such as Lck and Fyn. However,

a coordinated balance between kinases and phosphatases is required for appropriate T cell proliferation, activation and survival. When this balanced is disturbed in some way, syndromes such as inflammation, autoimmunity and leukemogenesis can occur. An overview of the characteristics and function of the kinases Lck, Fyn and Csk, and also of the phosphatase CD45 will be given in the next paragraphs.

4.2.1. Src family tyrosine kinases Lck and Fyn

Lck and Fyn are identical in their overall structure. They possess on the N-terminal amino acids that can bind to saturated fatty acids allowing their attachment to the inner leaflet of cell membrane; SH3 and SH2 domains that can mediate interactions and function as adapters; a tyrosine kinase domain; and a C-terminal negative regulatory domain. The main structural difference is the unique kinase domain in their N-terminal region which provides specific functions for each kinase. Moreover, different binding properties of the Lck and Fyn SH2 and SH3 domains can also give diverse functionally specificities to both proteins (193). These domains are involved in protein-protein interactions through the binding to phosphorylated tyrosines and proline residues, respectively. The unique domain of Lck intervenes in the association with CD4 or CD8. This association occurs within a di-cysteine motif of Lck and two cysteine residues present in the cytoplasmic tail of both CD4 and CD8 (194). Besides having a role in CD4/CD8 binding, the unique domain of Lck also regulates membrane association (195), and can influence substrate specificity (196) and interaction with regulatory phosphatases (197). Fyn's unique domain was shown to bind to the ε-chain of the CD3 complex, although at low stoichiometry (198).

It is well established that both Lck and Fyn are engaged to the TCR-CD3 complex, nevertheless their recruitment depends on their capacity to interact with different downstream targets and signaling effectors (193). Particularly, stimulation through anti-CD3 mAbs can induce Cbl tyrosine phosphorylation, a Fyn substrate, but not the phosphorylation of LAT, a ZAP-70 substrate (199). Moreover, activation of Fyn via CD3 results in Fyn-tyrosine mediated phosphorylation of PAG, a lipid raft (LR) resident protein and consequent sequestration of active Ras to LR that could induce T cell anergy (200).

Several genetic studies demonstrated that Lck and Fyn have a critical role in thymocyte development. When analyzing Lck knockout mice, a block in T cell development was observed, especially in the DN3 stage, where pre-TCR signaling is required to cross this developmental checkpoint (201). While DP are reduced ~10 fold, SP

are almost absent, which indicates an essential role for Lck in DN3 checkpoint and efficient pre-TCR signaling. By contrast, Fyn knockout mice did not show any significant difference in thymocyte subsets development, indicating that probably the lack of Fyn can be compensated by the presence of Lck (202). Nonetheless, a strong phenotype was observed in the Lck-Fyn double knockout, with an absolute block in the transition of DN3 to the DN4 stage, which on the other hand could indicate that Fyn can compensate for Lck at early stages of T cell development (203, 204).

Lck and Fyn are activated after TCR-pMHC engagement in a highly coordinated dance. This kinase activity regulation is achieved through conformational changes of SH3 and/or SH2 domains after binding to regulatory ligands (205) and phosphorylation/dephosphorylation of activatory vs inhibitory tyrosine residues (206). When the regulatory/inhibitory tyrosine residues of both Lck and Fyn (Y505 for Lck or Y528 for Fyn) are phosphorylated by C terminal *Src* kinase (Csk), the C-terminal region clasps on its SH2 domains and blocks the tyrosine kinase domain that becames inactive and in a close conformation (207). This inactive conformation is stabilized by SH3 domain interactions with polyproline helix II stretches between SH2 and kinase domains (208). However tyrosine 505 and related sequence (Y^{505}QPQP) is not optimal to interact with the SH2 domain, suggesting that the Lck tail would actively switch on and off to the binding pocket of the SH2, making this interaction unsteady (209, 210). Yet, Src kinases are generally in equilibrium between inactive and active state. Furthermore, binding of both SH2 and SH3 domains to intracellular ligands is of high affinity, which can induce the release of the inhibitory conformation (211).

Recruitment of Csk to the vicinity of Src family kinases is mediated by the adapter protein phosphoprotein associated with glycosphingolipid-enriched domains (PAG), also known as Csk binding protein (Cbp) (212). Curiously, the association of PAG with Csk is dependent on PAG phosphorylation by Fyn (213), indicating an interdependence of both kinases as they can regulate each other by activating a negative feedback loop. On the other hand, there is the tyrosine phosphatase CD45 which dephosphorylates the C-terminal residues of Lck and Fyn, having a fundamental role in maintaining an open active conformation of the kinases (214, 215).

Besides the inhibitory tyrosine residues, there are the activatory counterparts, critical tyrosine residues in both Lck (Y394) and Fyn (Y417) that facilitate the enzymatic activity and therefore are referred to as activatory tyrosines. The activatory tyrosine localizes in the “A loop” and can only be available for phosphorylation when the kinase domain is in an open conformation. Briefly, for activation of either Lck or Fyn, firstly ligand

binding to SH2/SH3 domains occurs, followed by dephosphorylation of the inhibitory tyrosine by CD45 which releases the kinase domain to an open state by displacement of A-loop helix, and then transphosphorylation of Src tyrosine kinases between themselves (216). Unlike Y505 of Lck, which can only be dephosphorylated by CD45 (217), several phosphatases, such as PEP, PTP-PEST, CD45 and SHP1, can inactivate the activatory tyrosine.

4.2.2. Csk a negative regulator

Csk is a non-receptor tyrosine kinase with SH3 and SH2 domains in its N-terminus and a kinase domain in the C-terminus (218). The main difference between Csk and Src kinases is the lack of an auto-phosphorylation site in the activation loop. Also, it has neither N-terminal fatty acylation nor C-terminal negative regulatory sites. In the active conformation, Csk SH2-kinase and SH2-SH3 linkers are tightly bound to the N-terminal lobe of the kinase domain, whereas in inactive molecules the SH2 domains are rotated in a manner that disrupts the linkage to the kinase domain. These observations suggest that both conformations of Csk exist in a dynamic equilibrium, and that the overall activity is regulated through binding of the SH2 domain to some other receptors (219). As Csk is a cytosolic protein, the translocation to the plasma membrane, nearby Src-family kinases (SFKs), is a critical step for its regulation. In resting T cells, the SH2 domain of Csk is bound to pY317 of PAG; this Csk-PAG complex represents the negative regulatory mechanism that controls activation/inhibition of both Lck and Fyn (212). When T cells are activated, dephosphorylation of the relevant tyrosine releases Csk to the cytosol, allowing SFKs to be activated.

4.2.3. The phosphatase CD45

CD45 is the most abundant protein tyrosine phosphatase (PTP) expressed on all nucleated hematopoietic cells. It contains extracellularly an N-terminal region with several lengths due to alternative splicing, followed by fibronectin-type repeats. The different isoforms derived from alternative splicing and differences in glycosylation can target the protein in a cell-specific manner. For instance, the CD45RABC isoform that comprises all exons is mainly expressed by naïve T cells, whereas the smaller isoform, CD45RO is present in effector-memory T cells (220). Expression of these isoforms is mostly related to the critical binding of heterogeneous nuclear ribonucleoprotein L (hnRNPL) which induces

exon skipping (221). The CD45 cytoplasmic region is composed of two PTP domains, where the first is enzimatically active (222) while the second seems to be inactive.

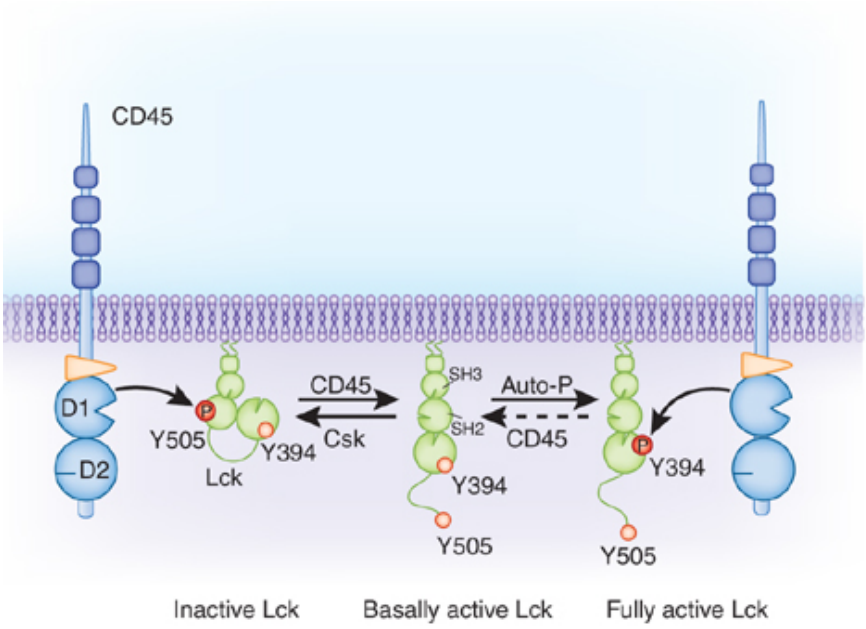

Figure 2 – Regulation of Src family kinases by CD45. In T cells, CD45 mediates dephosphorylation of the inhibitory C-terminal tyrosine residue of Lck (Y505). This occurs in resting T cells and disrupts the close conformation of Lck, thus augmenting the basal kinase activity of Lck. After autophosphorylation (auto-P) of another tyrosine (Y394) located in the kinase domain, Lck becomes fully active. CD45 can also, albeit less efficiently, dephosphorylate Y394, thereby restricting the extent of Lck activation. The tyrosine kinase responsible for phosphorylation of Y505 is Csk (Image taken from Rhee and Veillette (223)).

Analyses of T cells devoid of CD45 and CD45-deficient mice established the role of CD45 in T lymphocytes. Results from this deficiency are: blocked T cell activation and consequently TCR signaling and interference with T cell development in the thymus (224, 225). Such defects are especially related to a hyperphosphorylation of the inhibitory tyrosine residues of both Fyn and Lck, leading to a dysfunctional kinase (214, 226, 227). For this reason, CD45 positively regulates T cell activation and development by dephosphorylating inhibitory tyrosine residues of SFKs. The positive regulatory role of CD45 opposes to its negative enzymatic activities affecting T cell responses. Those include dephosphorylation of the activatory tyrosine residue of Lck (Y394) and the phosphorylated tyrosines within CD3 ITAMs (228, 229). This dual function allows CD45 itself to regulate a negative feedback mechanism in order to restrict continuous activation of SFKs in T cells (Figure 2). However, the question of how CD45 antagonistic activities are delivered on a single cell level remains unanswered.

5. Spatial organization of the T cell signaling platforms

5.1. Immunological synapse formation

The organization of membrane proteins at the vicinity of a T cell–APC contact was termed immunological synapse (IS) (230, 231). The word synapse, coined from the greek “syn-” (“together”) and “haptein” (“to clasp”), was first mentioned by Sherrington and colleagues in 1897. Michael Dustin suggested that the IS can be considered to have three functional layers (Figure 3), encompassing receptor interactions, signal transduction and cytoskeleton transport, as demonstrated for integrin-based focal adhesions (232). These layers provide a third dimension to the lateral organization of the immunological synapse into supramolecular activation clusters (SMACs) (233). Kupfer (231) defined the T cell and APC interaction as having a characteristic of bull’s-eye pattern with a central cluster of TCR-pMHC and the co-receptor CD28 engaging CD80/86 named central supramolecular activating complex (cSMAC), surrounded by a ring of the cognate integrin lymphocyte function-activated antigen 1 (LFA-1) and its immunoglobulin superfamily ligand intercellular adhesion molecule 1 (ICAM-1), defined as the peripheral SMAC (pSMAC). Additional studies showed that a number of transmembrane and cytoskeletal proteins were removed from the contact zone. The region formed outside the pSMAC seems to be rich in CD45, and was referred as distal SMAC (dSMAC) (234).

Formation of the IS happens over the course of minutes to hours and correlates with full T cell activation. Studies with supported planar bilayers revealed a similar structure of small and large adhesion molecules in activated T cell contacts, suggesting an IS (235). The use of these bilayers, in which high densities of pMHC complexes and ICAM are seeded, has given enormous details of molecular rearrangements in signaling structures, owing to its capacity to be imaged with total internal reflection (TIRF) microscopy. However, in these system cells form a cSMAC conformation in more than 90% of the cases, while physiological T cell-APC contacts occur with lower frequencies, varying from 30% measured by electron microscopy to 50-80% as observed by confocal microscopy (236).

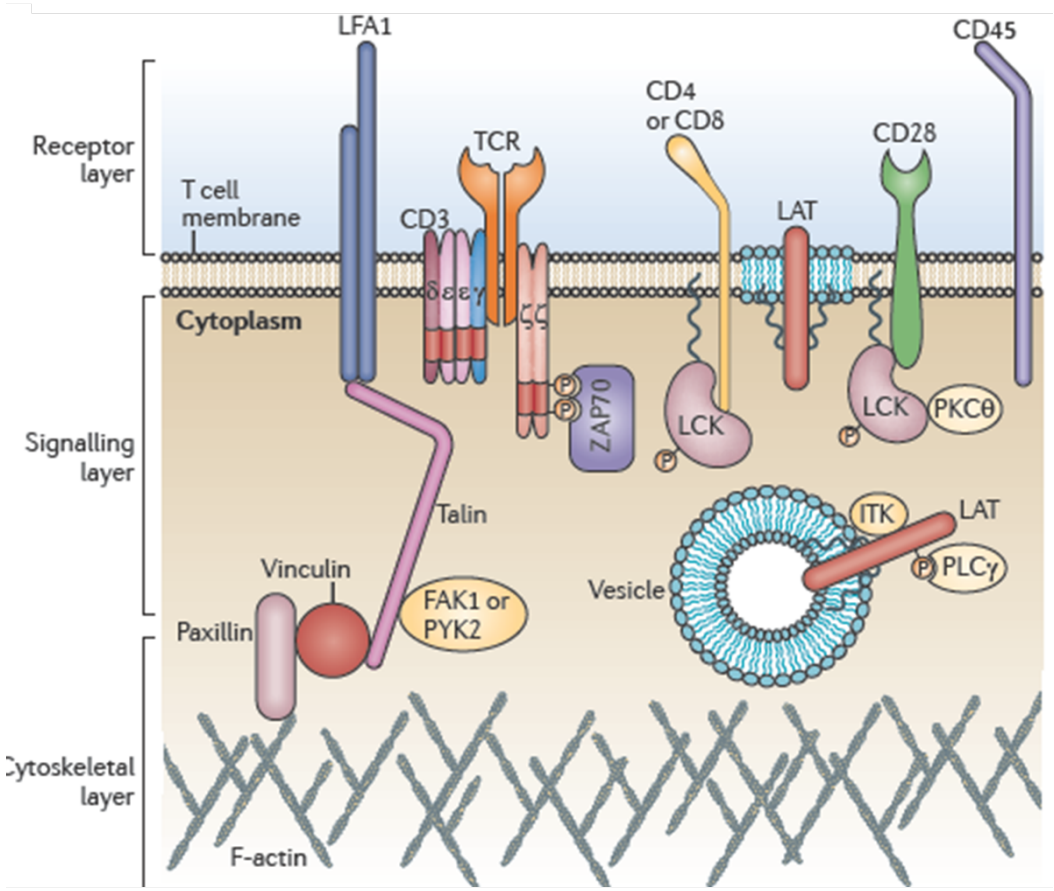

Figure 3 - Structure of the immunological synapse. The three layers on the T cell side of the immunological synapse are represented. The receptor layer contains the TCR–CD3 complex, CD4 or CD8, CD28 and LFA1. The signaling layer includes Lck, ZAP-70, Itk, PLCγ and PKCθ. The cytoskeletal layer contains F-actin, talin, paxillin, vinculin and focal adhesion kinase 1 (Fak1) or proline-rich tyrosine kinase 2 (Pyk2). The adaptor protein LAT is shown both attached to the plasma membrane and anchored to sub-synaptic vesicles. (Image taken from Dustin and Depoil (233))

TIRF microscopy revealed small T cell-APC microclusters of few hundred nanometers that coalesce to form larger structures in the cSMAC (134, 135). In lipid bilayers, TCR microclusters appeared in dSMAC and then move to the cSMAC region in an F-actin-dependent process (134, 135). Such discrepancies in topology between lipid bilayers and TIRF microscopy can indicate that each cell-cell contact is a small microcosmus and *in vivo* T cells are more robustly motile than *in vitro*. Increasing information is being achieved from advanced super-resolution imaging, but data interpretation is complex and there is much to learn.

The above-mentioned layers include the receptor layer, the signaling layer and the cytoskeleton layer (233). The first one comprises TCRs, adhesion molecules (like LFA-1),

co-stimulatory and co-inhibitory molecules, and co-receptors (233). Adhesion receptors can function in adhesion contexts beyond antigen recognition (237). An example is the CD2 and CD58 (pMHC independent) interaction of cytotoxic T lymphocytes (CTLs) with target cells (238). Co-stimulatory and co-inhibitory proteins can be defined as having a positive or negative role in TCR triggering; a good example is the CD28 (co-stimulatory) and CTLA-4 (co-inhibitory) pair, as explained in 3.2. The most dynamic area of research in recent years has been the study of inhibitory molecules, including programmed cell death protein 1 (PD1) and 2B4 (240), but investigation on this subject needs much detail.

The signaling layer comprises a tyrosine kinase cascade, a nuclear factor-κB (NF-κB)-activating oligomeric complex and ubiquitin-dependent signal termination (233). The TCR itself does not have intrinsic catalytic activity; however, as explained earlier, it is assembled as a multisubunit with CD3, which contains ITAMs that can be phosphorylated. After an intense exchange of information between kinases, adaptor proteins and others, transcription factors like NF-κB are activated. Signal termination is mediated in part by endosomal sorting complex required for transport proteins (ESCRT) that manage TCR dephosphorylation and cSMAC formation (241).

The cytoskeleton layer brings together three filament-forming proteins: actin, myosin II and tubulin (233). These filaments are coordinated by polarity network and receptor signaling (242). TCR couples to actin through adaptor proteins and the actin regulator Wiskott-Aldrich syndrome protein (WASP), an interaction important for T cell activation and normal IS formation (243). The TCR and B cell receptor (BCR) can both link to the microtubule motor dynein (244, 245).

5.2. The role of Integrins

Integrins are a group of type I transmembrane proteins that form heterodimers, each consisting of one α-subunit and one β-subunit; so far a total of 18 α-subunits and 8 β-subunits were described (246). They function as adhesion molecules and as receptors suited to transmit chemical and mechanical information from the external environment into the cell (a process termed “outside-in signaling”) (247).

A particular feature of these proteins is that they exist at the cell surface in an inactive form until they receive a stimulus from other receptors (via inside-out signaling). In addition, upon activation there is a dramatic conformation shift, from a bent, compact shape, to an extended, open one. Integrin activation happens when cytoplasmic proteins bind to the α- and β-subunit carboxy-terminal tail, these interactions stabilize the extended

conformation and provide associations with the cytoskeleton. In lymphocytes, stimulation by the B cell receptor (BCR) and the T cell receptor (TCR) also modulates integrin avidity (248).

Common integrins are leukocyte function-associated antigen 1 (LFA-1), Mac-1 and very late antigen 4 (VLA-4). The counter-receptors for LFA-1 are the intercellular adhesion molecules (ICAMs 1-5); Mac-1 has different types of ligands including extracellular matrix proteins such as fibronectin and fibrinogen and activated complement proteins such as iC3b. VLA-4 recognizes the vascular intercellular adhesion molecule, VCAM-1. LFA-1 is the best characterized integrin in the T cell context, it is important for cell arrest and migration on APC surfaces where its ligand is expressed, and consequently for the formation of the IS (233).

5.3. Membrane microdomains

The meeting place that encompasses T cell activation after engagement of the TCR with the pMHC is the plasma membrane. To accomplish a coordinated, specific and efficient signaling, a number of events need to be accurately correlated in time and space, which is normally achieved by segregation of signaling elements into an appropriate confinement. This membrane organization is complex and highly dynamic, that besides the involvement of immunoreceptor engagement, translocation of critical effectors to cell-cell contacts and assembling of signaling complexes, it also includes a sustaining cellular architecture. Protein segregation can occur within the plasma membrane into microdomains, also named lipid rafts (249). These platforms can be supported by a cytoskeletal network that regulates it structurally and functionally (242, 250, 251). The above-mentioned microdomains are liable to controversial lines of thought. There are those who defend that lipid rafts are specialized detergent resistant lipid ordered domains especially enriched of cholesterol, glycosphingolipids and GPI-anchored proteins, whereas others simple neglect the existence of them.

Such domains are particularly dynamic but with a very short lifetime (less than a second, sometimes even thought to be in the order of 1 ns) (252, 253). The properties of raft domains differ from stimulated vs resting cells. After cell stimulation, protein-protein interactions induce the formation of clusters promoting stable and larger rafts, their size can range from 10 to 20 nm and lifetime is increased to ~100 to 1000 s (254). Briefly, we can only call lipid rafts as a structure when they are stable formed after stimulation, otherwise they are dynamic and short lived (255). Detergent resistant microdomains were

essentially characterized to explain many events in TCR signaling, some of them are exemplified on chapter II, section 3.

6. BRET – A methodology to study protein-protein interactions

A complete map of the human interactome is the principal goal to be achieved in the field of proteomics. Protein-protein interaction (PPI) analyses are essential to comprehend the function and cellular processes in which proteins are involved. Several thousands of possible PPIs are thought to be part of the human interactome (256, 257). Numerous technologies have been developed to detect and analyze protein-protein interactions, some are more specialized in high throughput screens while others are restricted to individual approaches. An accurate knowledge of the proper technique to analyze those interactions is necessary in order to answer specific biological functions.

Among these technologies, bioluminescence resonance energy transfer (BRET) is of particular importance since it permits the detection of PPIs in living eukaryotic cells in which proteins can be expressed and folded in their physiological environment, allowing for suitable posttranslational modifications and correct sub-cellular compartment localization. The BRET system is based on the naturally occurring process resulting from non-radiative energy transfer between a donor (*Renilla* Luciferase) and an acceptor (e.g. GFP or YFP) (258), constituting the BRET pair. The main requisites for an appropriate efficiency on energy transfer ($BRET_{eff}$) are that the emission spectrum of the donor overlaps the excitation of the acceptor and the distance between both cannot exceed 100 Å. The bioluminescent protein isolated from *Renilla reniformis*, Luciferase, oxidizes its substrate, coelenterazine, emitting light in wavelengths that range from 400 to 510 nm. If an appropriate acceptor is in the surroundings, from 10 to 100 Å, it will be excited to a higher energetic state and energy transfer will occur, emitting photons with longer wavelengths. An optimized version of this technique, named $BRET^2$, uses DeepBlueC, an analogue of the natural substrate, with maximal emission peaking at 410 nm, and GFP^2, a UV-GFP variant, that can be excited at this wavelength and emits at 515 nm, giving a better spatial separation of about 100 nm, which facilitates distinction between both emissions (259).

Besides BRET, fluorescence resonance energy transfer (FRET) is another frequently used method, in which energy transfer involve two fluorophores but an outside energy source is needed to promote donor emission.

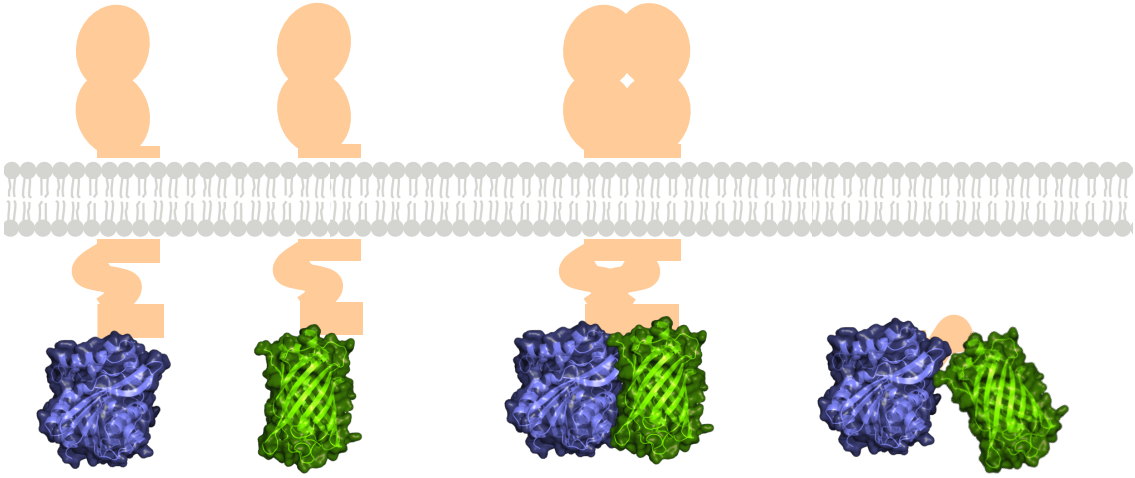

Figure 4 - A diagram of bioluminescence resonance energy transfer (BRET) used for a protein-protein interaction assay. One protein of interest (blue) is genetically fused to the donor luciferase RLUC and the other candidate protein (green) is fused to the acceptor fluorophore GFP. In the presence of the substrate, coelenterazine, RLUC emits luminescence (peak at 410 nm). Interaction between the two fusion proteins can bring RLUC and GFP close enough for BRET to occur, with an additional emission at a longer wavelength (e.g. peak at 515 nm). The interaction is shown in the middle panel by the two proteins together (blue and green). The third pair on the right represents the positive control, the ideal case of 100% resonance transfer, by using a construct where RLUC and GFP are fused.

The donor needs to be excited by an external light, whereas in BRET, oxidation of the substrate strongly reduces the signal to noise ratio. Also, in BRET there is no photobleaching or other optical effects, giving a particularly low background. This reduction on the background allows proteins to be analyzed at much lower concentrations being useful for the detection of weaker interactions. Proteins of interest are fused to either GFP[2] or *Renilla luciferase* (Rluc) and expressed in a suitable cell line as a BRET pair. If the proteins are in close proximity and interact, energy transfer will occur and both GFP and RLuc will give an emission signal. When protein-protein interactions are absent, only donor signals are emitted. Caution should be taken as background signals can derive from random interactions. The fact that both BRET an FRET use fusion proteins can be a disadvantage, as fusion can promote steric hindrance hampering wild type interactions.

There are several studies that use BRET analysis to study PPIs, these include screenings to search for PPI inhibitors (260), study dynamic GPCRs in living cells (261), analysis of proteases activities (262), among others. Generally the BRET methodology is employed to screen for clinical compounds to be used in biology or nanomedicine, although BRET is important to characterize protein dimer and/or oligomer formation.

Chapter II

Research Work

MOLECULAR CLONING AND ANALYSIS OF SSc5D, A NEW MEMBER OF THE SCAVENGER RECEPTOR CYSTEINE-RICH SUPERFAMILY

Carine M. Gonçalves, Mónica A. A.Castro, Telmo Henriques, Marta I. Oliveira, Vattipally V. Sreenu, Alexandre M Carmo

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Introduction

The scavenger receptor cysteine-rich superfamily (SRCR-SF) comprises a group of molecules with one or more protein modules sharing structural homology with the membrane distal extracellular domain of the macrophage type I scavenger receptor, a trimeric integral membrane protein found mainly on macrophages (263). SRCR proteins can consist exclusively of SRCR domains or may include other types of protein modules such as EGF, CUB, LCCL, or other domains. Proteins belonging to the SRCR superfamily are typically expressed in cells associated with the immune system (264), although some members are expressed in non-immune cells in various tissues and organs, such as the liver, kidney, placenta, stomach, brain and heart (16). SRCR domains have a compact fold consisting of a curved six/seven-stranded β-sheet cradling an α-helix (20, 265, 266).

The 100–110 amino acid polypeptide sequences of SRCR domains have highly characteristic patterns of cysteine residues that, along with the organization of the genomic sequences encoding each domain, allow SRCR domains to be divided into two groups. Group A SRCR domains are generally encoded by split exons and possess six cysteines forming three disulphide bonds, whereas the single exon encoded group B modules have eight cysteine residues forming four disulphide bonds per domain (16). Remarkably, although as stated some SRCR family proteins contain non-SRCR domains, no known proteins have both group A and group B SRCR domains.

In contrast to group A-containing proteins, which are present in phyla comprising the most primitive metazoa, *i.e.* Porifera (267, 268), to vertebrates, group B SRCR domains have only been found in the vertebrates (16). Despite their generally being a very high degree of homology between paralogous proteins in the same species, and between orthologs across species, there is, as yet, no unifying biological function for the SRCR superfamily. Indeed, the “scavenger” designation may have been an inappropriate choice: the two forms of the macrophage scavengers are functionally indistinguishable (263), yet only scavenger receptor I contains a “scavenger” SRCR domain, suggesting that the module may not have any scavenging capacity at all.

Of all the members of the SRCR superfamily, the lymphocyte cell surface antigens CD5 and CD6, which function in T and B cells and contain three extracellular SRCR domains, have received the most attention (21, 269). CD5 and CD6 co-associate with each other at the surface of T cells (54, 84), and are implicated in T cell receptor and CD2 function (45, 46, 52, 70), whereby they influence the initial steps of T cell activation. With the extensive characterization of the interaction of CD6 with its antigen presenting cell-expressed ligand, CD166 (270), and the identification of a plethora of putative binding

partners for CD5 (41-44), at least some SRCR domains are generally assumed to participate in intercellular communication *via* protein–protein interactions, and to therefore have a role in cellular differentiation and activation. In addition to CD5 and CD6, the group B SRCR family presently contains five other proteins. The soluble protein Spα contains three SRCR domains and is expressed exclusively by cells of lymphoid origin (271). DMBT1, which was identified on the basis of its deletion in a medulloblastoma cell line and is the largest member of the family, contains 14 SRCR domains separated by SRCR-interspacing domains (28). CD163 (272) and M160 (CD163L1) (273), which were both identified in human monocytes, are considered a subgroup of the SRCR group B molecules. The latest identified member, S4D-SRCRB, is a soluble protein with four group B domains that is expressed broadly (32). For these molecules no definitive function has been established. Recently, however, it has been suggested that Spα (274), DMBT1 (275), CD163 (276), and even CD5 (277) and CD6 (83) are capable of detecting microbe-associated molecular patterns, and could bind and clear bacteria or fungi, raising yet another scavenger-like role for this group of molecules. These developments notwithstanding, SRCR superfamily proteins may prove to have very diverse functions, to the extent that the structural properties of the highly conserved SRCR domains may be the only unifying feature of the family.

We reasoned that a clearer understanding of their evolution and functions would emerge when all the members of this protein family had been identified. The advent of the human genome sequence allowed us to find, using a bioinformatics-based approach, a new SRCR protein, which we have called Soluble Scavenger with 5 Domains, SSc5D. We describe the expression and likely structural features of this new protein, and consider the evolutionary relationships within the family.

Materials and Methods

Cells and Cell Lines

Cell lines used were Jurkat E6.1 of T-lymphocyte origin (278), the monocytic leukemia cell line THP-1 (279), and the B cell lines Kw-Bcl and MEC-1 (280). Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 1mM sodium pyruvate, 2mM l-glutamine, penicillin G (50 U/ml) and streptomycin (50µg/ml) all from Invitrogen. Peripheral blood mononuclear cells (PBMC) were isolated from buffy-coats of healthy volunteers by centrifugation over Histopaque-1077 (Sigma–Aldrich). Cell sorting using a FACSAria (Becton Dickinson) was then performed to select monocytes

and different lymphocyte subpopulations ($CD4^+$, $CD8^+$, and $CD19^+$). Conjugated mAbs used for cell sorting were multimix dual color anti-human CD4-FITC + CD8-RPE (MT310 and DK25; Dako), CD11b- RPE (MEM-174) and CD19-APC (LT19), both from Eurobiosciences.

RNA preparation, reverse transcription and Northern blot analysis

Total RNA was isolated from non-stimulated human primary cells, and from cell lines using both TRIzol (Invitrogen) and the total RNA purification RNeasy Midi Kit from Qiagen. RNA was quantified using a NanoDrop ND-1000 UV–Vis Spectrophotometer. The A_{260}/A_{280} ratio of the RNA was always higher than 1.7. First strand cDNA was synthesized using 1 µg of total RNA and 0.5 µg oligo-dT primer with the reverse transcriptase (RT) Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. A control without RT was also performed. To generate a SSc5D probe, a 2500 bp PCR product comprising the 5' end of the SRCR domain 1-coding sequence through to the 3' end of the SRCR domain 5-coding sequence was obtained by PCR, using primers SRCRD1 and SRCRD5 (Table 1), with the following conditions: 95 ºC for 1 min and 25 cycles of 95 ºC for 30 s, 60 ºC for 30 s, 68 ºC for 2 min 30 s, and cloned into the TOPO TA vector (Invitrogen).

The insert was excised with *EcoR*I and *Xho*I (present at the multiple cloning site of the vector and at the domain 5-coding sequence, respectively), gel-purified and labeled with Amersham Ready-To-Go DNA Labelling Beads (-dCTP) (GE Healthcare). The radiolabeled probe was then hybridized to a First-Choice Northern Human Blot I membrane (Ambion) in UltraHyb buffer (Ambion), washed to a stringency of 0.1×SSC, 0.1% SDS at 42 ºC, and exposed at −80 ºC to X-ray films (Kodak) for different periods of time. Northern blot was normalized with a supplied β-actin probe

TABLE 1

Oligonucleotides used in this study

Primer name	Sequence
Cloning primers	
5GSP1	5' GGAGTTAGCCACACGCTGA 3'*
5GSP2	5' GCGCTCAACAGCCTGGAT 3'*
3GSP1	5' CTCATCTTGACAAGCCCTG 3'
3GSP2	5' TGTGACCCTCTCCAGGATT 3'
ATG	5' TGGCTGCAACCATGAGGGTCTT 3'
STOP 4.8kb	5' AATCCTGGAGAGGGTCACA 3'*
STOP 3kb	5' GCTAGGGAGGCGCTGCAA 3'*
SRCRD1	5' TAGTAGGAATTCACCGTGTGTGATGACGGCT 3'
SRCRD5	5' GTGACCACGAGGAAGACGT 3'*
qPCR primers	
hACTB F	5' GGATGCAGAAGGAGATCACTG 3'
hACTB R	5' CGATCCACACGGAGTACTTG 3'*
Exon 14 4.8kb F	5' ACTGACCCAGGTCGTGGAAC 3'
Exon 14 STOP R	5' TAGTGGTGGTTGGTGCAGG 3'*
3Kb F	5' ACACAGACTATGACGATTATCC 3'
3Kb R	5' AGCATCACCTCTCGGAACTC 3'*

All the oligonucleotides used to amplify genes are shown, with primers with the reverse orientation of the gene sequence marked by an asterisk.

cDNA Cloning

The 5' and 3' untranslated regions (UTR) of the *SSD5D* transcript were identified using 5' and 3' rapid amplification of cDNA ends (RACE). 5' RACE was performed using RACE-ready cDNA from human spleen (Applied Biosystems/Ambion). Briefly, a first PCR was performed on a cDNA library from spleen with a gene-specific primer (5GSP1, Table 1) and the supplied 5' RACE outer primer using Advantage 2 polymerase Mix (Clontech) and the following cycling conditions: 3 min at 95 ºC, and 35 cycles of 95 ºC for 30 s, 55 ºC for 30 s, 68 ºC for 1 min, and a final extension step at 68 ºC for 5 min. This was followed by a nested PCR with a second gene-specific primer (5GSP2, Table 1) and the supplied 5' RACE inner primer, using a 1/100 dilution of the first PCR as template, and the same cycling conditions. The identification of the 3' end sequences of *SSC5D* was obtained by

3' RACE, using the same kit conditions described before, except that the extension time of the PCR reactions was increased to 3 min. The primers used (3GSP1 and 3GSP2) are shown in Table 1. All the resulting products were cloned into the TOPO TA vector and sequenced in both directions with the universal primers 'SP6' and 'T7 promoter'. The full-length coding cDNA was cloned by long distance PCR using primers at the initiation and termination codons (ATG and STOP primers, Table 1) and the enzyme Expand High fidelity PCR System (Roche Applied Science) with the following conditions: 2 min at 92 ºC, 3 cycles of 92 ºC for 10 s, 64 ºC for 30 s, 68 ºC for 10 min, and then 5 and 30 cycles with an annealing temperature of 62 ºC and 60 ºC, respectively.

PCR and real-time PCR analysis

We assessed the expression of SSc5D in different cells and cell lines of hematopoietic origin by PCR amplifying the mRNA transcript from the sequence corresponding to the beginning of the first SRCR domain until the end of the fifth domain-coding sequence, using the primers SRCRD1 and SRCRD5 with the following conditions: 95 ºC for 1 min and 25 cycles of 95 ºC for 30 s, 60 ºC for 30 s, 68 ºC for 2 min 30 s. To screen for the basal expression patterns of the two different isoforms of SSc5D by real-time PCR in $CD4^+$ and $CD8^+$ T cells, monocytes and B cells, two distinct samples were tested with primer pairs specific for each isoform (primers used are shown in Table 1). The expression study was performed in 96-well plates on an iQ5 real-time PCR detection system (Bio-Rad). A master mix was prepared for each plate and each quantitation target in 20µl reactions, with final concentrations of 1× iQ Sybr Green Supermix (Bio-Rad) and 200nM (4.8 kb isoform) or 300nM primers (ActB reference gene). The PCR program was initiated at 95 ºC for 3 min to activate iTaq DNA polymerase, followed by 40 thermal cycles of 10 s at 95 ºC, 20 s at 59 ºC or 59.5 ºC (respectively for 4.8 kb and ActB primer sets) and an extension time of 20 s at 72 ºC. The melting curve was generated using a temperature range of 65–95 ºC. Each sample was analyzed in triplicate wells. In addition, all the reactions were further subjected to electrophoresis on 2% agarose gels stained with ethidium bromide to confirm expected PCR products. For the standard curves a genomic fragment comprising the desired target region was amplified, purified with the QIAquick PCR purification kit (Qiagen) and the concentration of the PCR products was measured with the NanoDrop ND-1000 UV–Vis Spectrophotometer. A ten-fold serial dilution was made and used for generation of the standard curve.

Serial analysis of gene expression

SAGE data were normalized and compared as described (281). The 15 leukocyte libraries interrogated included LongSAGE libraries from resting and activated $CD4^+$ T cells (282) and from activated $CD8^+$ T cells (283); standard SAGE libraries from resting $CD8^+$ T cells and ex vivo sorted NK cells (281), and libraries downloaded from 'Blood SAGE'(284) for resting and activated B cells, granulocytes, resting and LPS-treated monocytes, M-CSF and GM-CSF stimulated macrophages, Langerhans cells, immature and mature dendritic cells. The 15 libraries derived from other tissues were downloaded from GEO (285) and represented the following tissues: cerebellum, thalamus, astrocytes, colon, heart, kidney, liver, lung, muscle, retina, prostate, cervix, ovary, stomach and vascular endothelium.

In vitro translation analysis

A DNA template was amplified for SSc5D-s (3 kb isoform) and cloned into the pcDNA3 vector (Invitrogen). The recombinant plasmid was purified using a Qiagen plasmid preparation kit, and the *in vitro* transcription and translation reactions were performed using the TNT T7 coupled reticulocyte lysate system as recommended by the supplier (Promega). Two micrograms of the plasmid DNA template were used for transcription and the protein was translated in each 50 µl reaction in the presence or absence of 40 mCi of [^{35}S]-methionine (1000 Ci/mmol) (Amersham) at 30 ºC for 90 min. The *in vitro* translation reaction was stopped by the addition of SDS sample buffer and the translated protein was separated by 7.5% SDS-polyacrylamide gel electrophoresis.

Alignments and phylogeny

Alignment of SRCR domains was performed using ClustalW2 at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/; (286)). NCBI reference sequences for the proteins used in the multiple alignments are: NP 006716.3 (CD6), NP 055022.2 (CD5), NP 004235.3 (CD163), NP 777601.2 (CD163L1-M160), NP 005885.1(CD5L-Spα), NP 015568.2 (DMBT1), and NP 542782.1 (S4D-SRCRB). We have used improvised maximum likelihood (ML) method (287) to carry out phylogenetic analysis. Sequences were aligned with ClustalW and gaps were removed from the final alignment. The Dayhoff substitution model was selected to calculate amino acid substitution rates. Reliability for internal branches was evaluated using the bootstrapping method (100 bootstrap replicates).

Results and Discussion

Cloning of SSc5D, a new member of the scavenger receptor cysteine-rich superfamily

We performed a systematic search for new members of the SRCR-SF in the completed human genome sequence by interrogating the draft genome sequence using TBLASTN 2.2.20+ (288) (http://www.ncbi.nlm.nih.gov/BLAST). We screened for new sequences exhibiting similarity with any or all of the SRCR domains comprising the seven known SRCR superfamily proteins (see material and methods for accession numbers of the test sequences). We reasoned that, for a given TBLASTN run, *bona fide* new SRCR domains would have smaller *E* values than the best matches of the search sequence with group A SRCR domains. According to this criterion, the search identified the sequences encoding domains within already known and characterized proteins, *i.e.* CD5, CD6, Spα, S4DSRCRB, DMBT1, CD163 and M160, but also a cluster of five new potential SRCR domains that are most similar to the SRCR domains of S4D-SRCRB and DMBT1 (Fig. 1).

The sequences encoding the five putative new domains mapped to the same strand in Chromosome 19 and within 11.5 kb of each other, suggesting that they belong to a single new gene. We decided to screen by RT-PCR for the presence of the putative new transcripts in cDNA samples from a panel of different cells and cell lines of hematopoietic origin, using oligonucleotides specific for the 5' end of SRCR domain 1 and the 3' end of domain 5. PCR amplifiable sequence was present in total PBMC, as well as in primary macrophages, in the monocytic cell line THP-1 and in the T cell line Jurkat E6.1, however it could not be detected in the B cell lines Kw-Bcl and MEC-1 (Fig. 2). The PCR product was approximately 2.5 kb in length, substantially larger than the prediction for just the SRCR domains, suggesting the existence of protein-encoding sequence between and/or beyond the scavenger domains.

To determine transcript size and tissue distribution we next performed a Northern blot analysis of poly (A)-rich RNA from human tissues using a pre-manufactured membrane (Ambion). The blot was hybridized with a cDNA probe of 2.5 kb prepared from the PBMC-derived PCR product described above, and a prominent transcript of nearly 5 kb was identifiable in placenta, spleen and colon, and more weakly in lung, heart and kidney, possibly reflecting the presence of blood contamination (Fig. 3). An additional transcript of ~3 kb could be seen in the samples from placenta, heart, lung and spleen, and a further 3.5–4 kb mRNA species in spleen, detected as a smear. A smaller band of ~2 kb present in all 10 lanes and whose intensity varied with the levels of α-actin, is assumed to be an artifact.

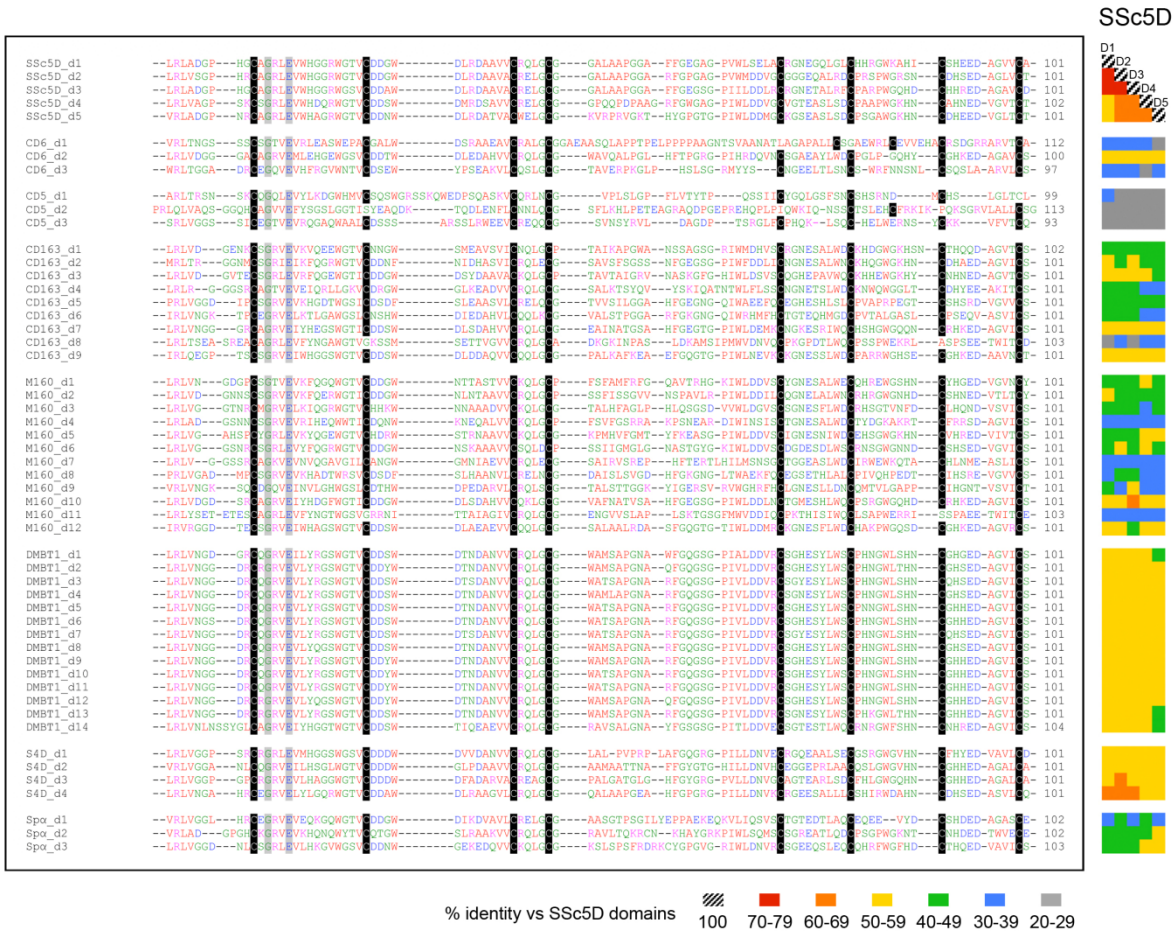

Figure 1 - Sequence alignment of SRCR domains from group B SRCR superfamily members. Amino acid sequences of SRCR domains of SSc5D, CD6, CD5, CD163, M160, DMBT1, S4D and SPα were aligned using ClustalW. Amino acid side chain color codes are: Red, small + hydrophobic (including aromatic except Tyr) side chains; Blue, acidic; Magenta, basic; Green, hydroxyl + amine + basic except Gln; Grey, others. Conserved cysteine residues involved in intrachain disulphide bonds are highlighted in a black background. Residues 100% conserved in all sequences analyzed are also marked, namely a Gly residue (light grey shaded), typically at position +12 from the N-terminus of the domain, and a Glu residue (grey shaded) at position +15. Each one of the 5 Domains of SSc5D was compared with every single domain of the full set of SRCR group B proteins present in the human genome. Similarities between domains are shown in the color graph displayed in the right, with the highest similarities in red (70–79% identity), and the lowest in grey (20–29%).

We used GENSCAN (289) (http://genes.mit.edu/GENSCAN) to predict the genomic structure of the putative new gene, and to identify start and stop codons consistent with the expression of a 5 kb transcript encoding all five SRCR domains. We designed oligonucleotides overlapping with these start and stop codons, and used them as 5' and 3' primers, respectively. Performing long distance PCR using cDNA from human spleen, we obtained a PCR product containing the entire coding region. To identify the 5' and 3' untranslated regions (UTR) of the putative new gene we performed 5' and 3' rapid

amplification of cDNA ends (RACE) using gene-specific primers hybridizing in the exons containing the start and stop codons, respectively.

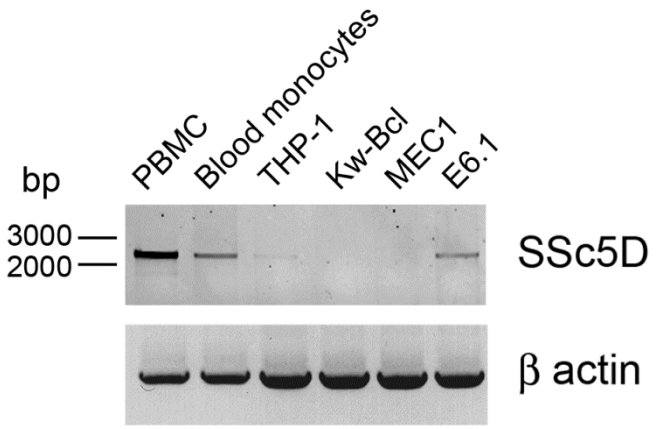

Figure 2 - RT-PCR analysis of *SSc5D* expression in blood cells and cell lines. Cells analyzed were peripheral blood mononuclear cells (PBMC), purified blood monocytes, the monocytic cell line THP-1, and lymphocytic lines of B (Kw-Bcl and MEC-1) and T (E6.1) cell origin. The 2.5 kb product includes all the cDNA sequences from the 5'end of the coding sequence of the first SRCR domain of SSc5D until the 3' end of the SRCR domain 5-coding sequence. A control PCR experiment was run using specific primers amplifying a β-*actin* product.

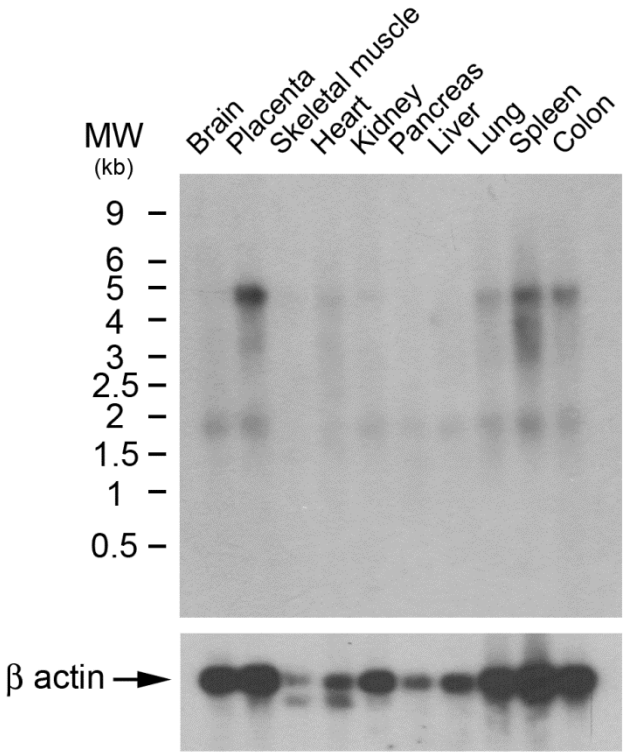

Figure 3 - Analysis of *SSC5D* expression by Northern blotting. A radiolabeled probe containing the nucleotide sequences encoding the 5 SRCR domains and connecting sequences of SSc5D was hybridized to a First-Choice Northern Human Blot I membrane (Ambion), containing poly (A)+ mRNA from human tissues. A hybridized mRNA species of 4.8 kb is highly expressed in placenta and spleen, and also detected at lower levels in colon, and more weakly in lung, heart and kidney. mRNA species of smaller sizes are also evident in spleen, placenta, heart and lung. A β-actin radiolabeled probe was subsequently hybridized with the membrane.

The PCR and RACE products were sequenced in both directions. The fully mature mRNA encoding the new molecule, which we have called SSc5D (GenBank accession number EU699476), is 4833 bp long and contains an open reading frame of 4722 bp encoding a polypeptide chain of 1573 amino acids (Fig. 4). This ORF encodes a mature protein with a molecular mass of *M*r 165,708 Da (predicted by ExPASy: http://www.expasy.org/cgibin/pi; (290)). The predicted SSc5D protein sequence contains an N-terminal signal peptide of 17 amino acid residues and a signal peptidase cleavage site at position 16/17 (predicted by SMART; http://smart.embl-heidelberg.de/). Three Asn-X-Ser/Thr sequons were found, only one of which is predicted to be an N-glycosylation site according to Net-NGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). The five SRCR domains each contain eight cysteine residues spaced as in other SRCR group B domains (264). Since no transmembrane region could be predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/; (291)), we conclude that SSc5D is likely to be a secreted protein.

Several SRCR members are expressed as alternatively spliced isoforms, some of which result from the skipping of individual SRCR domain-encoding exons (32, 66, 292), or from the differential inclusion of cytoplasmic domain-coding exons (60, 273). In the case of CD6, skipping of exon 5 results in the production of a CD6 isoform lacking SRCR domain 3, the ligand binding domain, that no longer traffics to the immunological synapse upon APC presentation of antigen (66). No other major function has been assigned to any of the isoforms resulting from the alternative splicing of other SRCR family proteins.

In order to determine whether the existence of the different transcripts identified in the Northern blot analysis was due to alternative splicing, we performed RT-PCR analysis of transcripts encoding SSc5D SRCR domains 1–5, but found no evidence of alternative mRNA isoforms likely to encode proteins lacking any of the SRCR domains. We also addressed the possibility that there was alternative splicing downstream of domain 5, by performing a 3'RACE analysis using gene-specific primers hybridizing at SRCR-d5 (GSP1 and GSP2; Table 1). A 3' RACE product was obtained and sequenced, which revealed a new stop codon and 3' UTR region consisting of 121 bp, in addition to a poly (A) tail located 17 bp downstream of the consensus pA signal sequence, AATAAA. The transcript for this new isoform, SSc5D short (SSc5D-s; GenBank accession number EU699477), is 3016 bp long, and contains an ORF of 2862 bp that encodes a protein of 953 aa (Fig. 4) with a molecular mass of 102,320 Da (predicted by ExPASy; (290)).

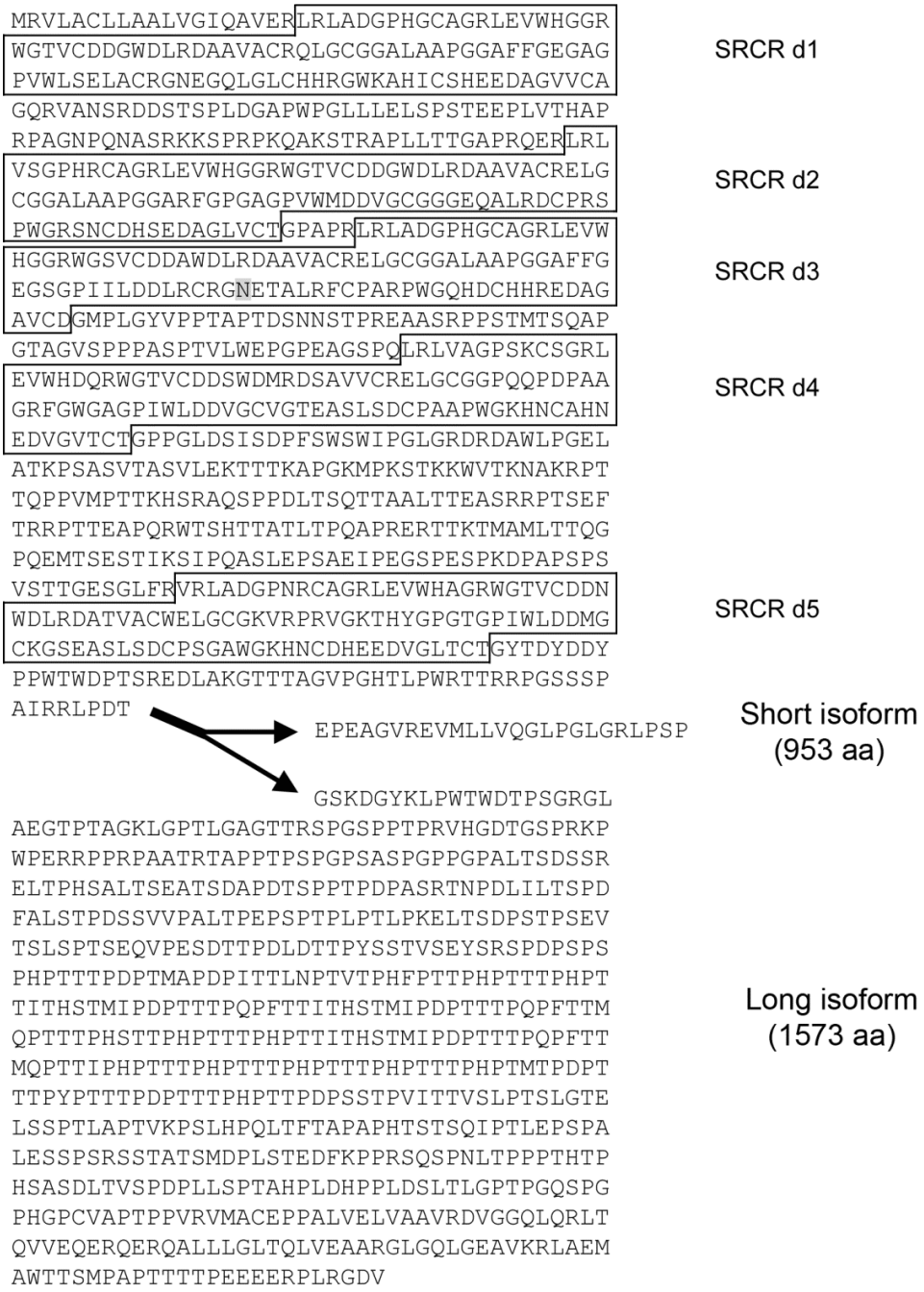

Figure 4 - Amino acid sequence of SSc5D. The amino acid sequences of the two isoforms of SSc5D were inferred from the cloned cDNA sequences. Individual SRCR domains are boxed. A predicted N-glycosylation site in the SRCR domain 3 is grey shaded. The first 923 amino acids are common to both SSc5D isoforms, and indicated in separate groups are the downstream sequences, Gly930 to Val1573, of full-length SSc5D, and Glu930 to Pro953 of the short SSc5D-s isoform.

SSc5D gene expression analysis

Our results were suggestive of the existence of a relatively abundant 4.8 kb mRNA species of *SSC5D* in monocytes/macrophages and T lineage cells as well as in placenta,

with the 3 kb species being expressed at substantially lower levels. We used real-time PCR, to study the expression of each of the two mRNA species in freshly isolated monocytes, $CD4^+$ and $CD8^+$ T-lymphocytes, and B lymphocytes. Assaying for the long isoform, it was evident that *SSC5D* is well expressed in monocytes and $CD4^+$ T cells, and at lower levels in $CD8^+$ T cells, whereas there is no detected expression in B-lymphocytes (Fig. 5). Using a set of primers specific to exon 13a, which would allow detection of the short isoform, *SSC5D-S*, we were unable to detect expression in any of the assayed primary cells (not shown). It is possible, therefore, that SSc5D-s is expressed in different stages of development or activation, or during immune responses.

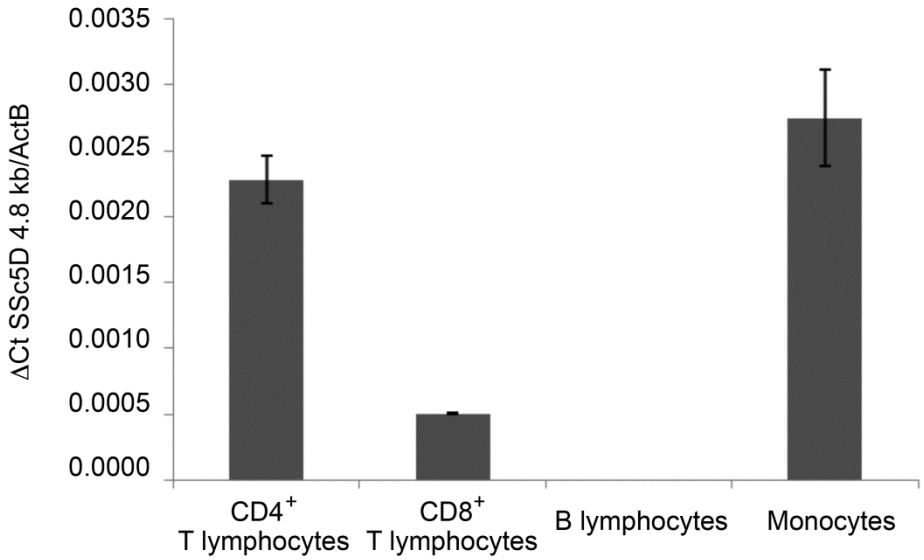

Figure 5 - Analysis of *SSC5D* expression by real-time PCR. Expression of *SSC5D* was analyzed on mononuclear cells, separated into different subpopulations, including $CD4^+$ and $CD8^+$ T-lymphocytes, B-lymphocytes, and monocytes/macrophages. Expression was highly detected in monocytes and $CD4^+$ T cells, and at lower levels in $CD8^+$ T cells, but was undetected in B-lymphocytes. Mean values and standard deviations are from 3 independent experiments.

We also screened in-house and publicly available transcriptomic data accessible in the form of serial analysis of gene expression (SAGE) libraries for the presence of tags derived from the two forms of SSc5D transcripts, but the analysis was inconclusive. We had access to 15 libraries from leukocyte-derived libraries, and 15 libraries from other tissues. The only SAGE tag found at a significant level in any of the libraries was not the 3' most tag for either splice variant (as would be expected) and matches an additional gene (PHD finger protein 1, PHF1). LongSAGE distinguishes between these two genes, and the only LongSAGE libraries available (from resting and activated $CD4^+$ T cells; (282)) contain the long tag from PHF1 not SSc5D. This suggests that an alternative tag may

exist for SSc5D that we have yet to identify. Such a tag could be generated by, among other things, alternative downstream polyadenylation of the transcript

Genomic organization of the gene encoding SSc5D and phylogenetic analysis

The *SSC5D* gene is located on chromosome 19q13.4. The 5' end of *SSC5D* maps 925 bp downstream of the 3' end of *NAT14* (*N*acetyltransferase 14), whereas the 3' end of the 4.8 kb isoform lies 9 kb upstream of the 5' end of *SBK2*, encoding SH3-binding domain kinase family, member 2. Alignment of the assembled SSc5D cDNA with the genomic sequence reveals that *SSC5D* has 14 exons distributed over 30.6 kb (Fig. 6).

The shorter isoform has 13 exons distributed over 15.8 kb. The exons range in size from 25 bp to 1.7 kb in length, separated by introns of 73 bp to 11.8 kb. Exon 1 comprises the 5' UTR and encodes, with exon 2, the putative signal peptide. Exons 3–11 encode the SRCR domains and interdomain regions. Each SRCR domain is encoded by single exons, as expected for SRCR group B members, and these vary from 309 to 318 bp in length. Exon 13a contains the stop codon for the short isoform. When skipping of this exon occurs, transcription proceeds to exons 13 and 14, the latter containing the stop codon and the 3' UTR of the long isoform and constituting the longest exon of the gene. All acceptor and donor splice sites conform to the AG/GT rule (293).

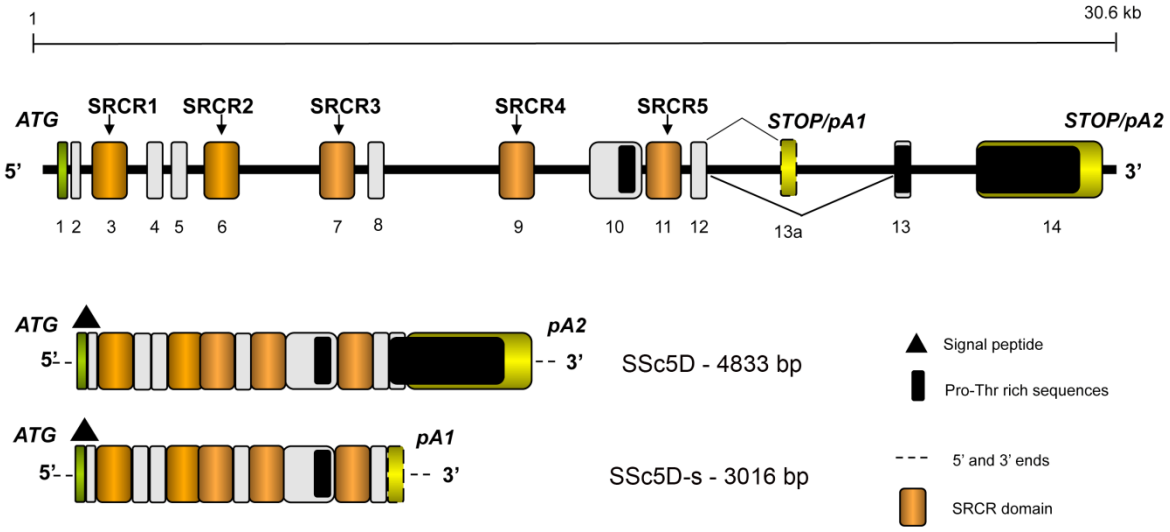

Figure 6 - Genomic organization of the SSC5D gene. The gene spans over 30.6 kb in chromosome 19q13.4. Coding regions are represented as boxes, and introns as connecting lines. SRCR domains are in orange, internal exons in grey, the coding regions containing the stop codons are in yellow, and that containing the start codon is in green. Black areas represent proline and threonine-rich sequences. The localization of the signal peptidase cleavage is indicated by a black triangle. Skipping of exon 13a is indicated, giving rise to full-length SSc5D.

The *SSC5D* locus maps in a telomeric region of chromosome 19, and gain or amplification of this region, particularly 19q13, has been associated with increased ovarian cancer incidence (294), and with multiple myeloma (295) or primary megakaryoblastic leukemias, and has been observed in acute myeloid leukemia cell lines (296). Conversely, loss of 19q13.4 has been reported in pancreatic carcinomas (297). There are also reports of cases of cerebellar ataxia and mental retardation connected to abnormalities in this region (298). However, given that SSc5D is not expressed in the brain, there is at present no strong link connecting SSc5D directly to neural functions.

Structure of the SSc5D protein

We wanted to assess whether SSc5D could be expressed *in vitro*, which would imply that the polypeptide is processed and that it folds efficiently. For this we used the short isoform, as it is more manageable and because it contains all five SRCR domains. We cloned the cDNA of SSc5D-s into an expression vector and performed *in vitro* translation using a TNT reticulocyte lysate system kit, obtaining a protein product of approximately 100 kDa, as predicted for the SSc5D-s isoform (Fig. 7), and also some smaller products, probably resulting from incomplete translation or from degradation of the 100 kDa species.

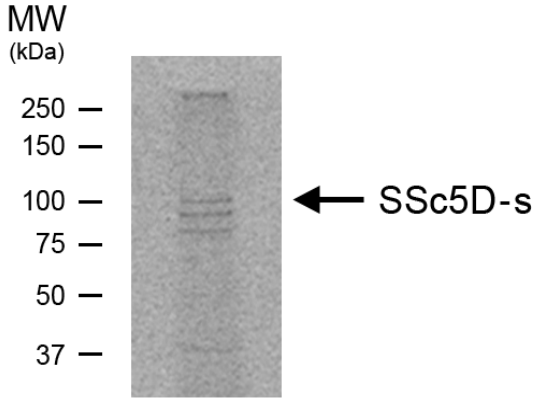

Figure 7 - *In vitro* expression of SSc5D-s. A cDNA template encoding SSc5D-swas cloned into pcDNA3 vector, and the construct was used in transcription and translation reactions using the TNT T7 coupled reticulocyte lysate system. The protein was translated in the presence of [35S]-methionine, run on SDS-PAGE and visualized by autoradiography

The N-terminal half of SSc5D is predicted to contain the five SRCR domains, separated by intervening sequences. A distinctive feature of the C-terminal portion of SSc5D is that it contains an extended series of repeats of six amino acid sequences rich in proline and threonine residues, *e.g.* PDPTTT or PHPTTT, reminiscent of similar sequences present in mucins (299). We therefore investigated the likely O-linked glycosylation content of this part of the molecule and of the entire protein, using NetOGlyc

3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/; (300)). As can be seen in Fig. 8, the C-terminal domain of SSc5D, including the sequences encoded by exon 13 and a large part of exon 14, encompasses numerous potential O-glycosylation sites, suggestive of a structure similar to that of highly O-glycosylated mucins. In the C-terminal part of SSc5D there are 144 potential O-glycosylation sites in a 476-long amino acid stretch, *i.e.* approximately three O-glycans for every ten amino acids. Considering a degree of extension of 2.5Å per residue, based on examples of submaxillary gland mucins (2.5 Å/aa; (301) or the stalk region of CD8 (2.6 Å/aa; (302), the estimated length of the O-glycan-rich C-terminal part of SSc5D would be close to 120 nm. Other stretches of SSc5D that are potentially glycosylated are within the linker regions between SRCR domains 3 and 4, and between domains 4 and 5. Overall, the dimensions of full-length SSc5D are likely to be over 250nm (Fig. 9), towards which the scavenger domains can only make a very small contribution, since the distance between the N- and C-termini of SRCR domains is only 3–4nm (20, 265).

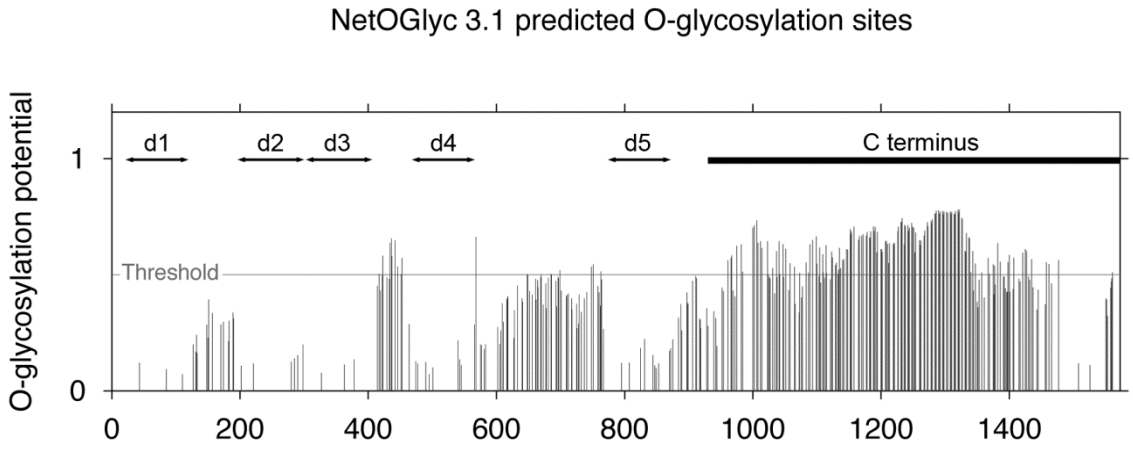

Figure 8 - Predicted O-glycosylation sites in SSc5D. Except for the 5 SRCR domains, all sequences of SSc5D have an O-glycosylation potential above background. The C terminal region of SSc5D, which includes sequences encoded by exons 13 and 14, is putatively extensively glycosylated, with 144 potential O-glycosylation sites in a 476-long amino acid stretch, *i.e.* approximately 30% of amino acids with a glycosylation potential above the calculated threshold.

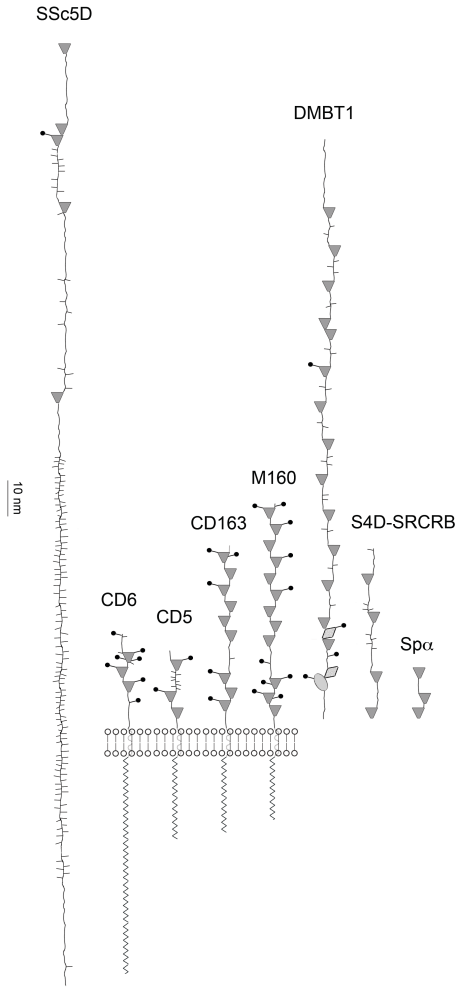

Figure 9 - Model of SSc5D comparison with all known class B SRCR molecules. SRCR domains (shown as grey trapezoid/heart shaped) are tentatively drawn to scale, according to the X-ray data described for the SRCR domain of Mac-2 binding protein, and of the third SRCR domain of CD5 (20, 265). The representation of CUB (diamond shaped) and Zona Pellucida (oval) domains of DMBT1 and the relative position of their N- and C-termini are based on the data described for the CUB domains of two sperm adhesins and of the C1s (303, 304), and the Zona Pellucida domain of ZP3 (305). Unstructured amino acid sequences are drawn as having a degree of extension of ~2.5Å per residue (301, 302). O- and N-linked glycans are represented as short lines and lines/circles respectively, also roughly to scale.

Phylogenetic analysis

Alignment of the full amino acid sequence of SSc5D with the predicted rat and mouse orthologs identified by BLAST (Fig. 10) revealed 68.8% identity between human and rat, and 67.5% identity between the human and mouse protein sequences. However, in comparisons involving the SRCR domains only, the homology between species increases dramatically: the average identity per domain is 95.1% between human and mouse, with the lowest being 92% (SRCR domain 1) and the highest 97% (SRCR domain 3), whereas between human and rat the average identity per domain is 95.9%, reaching 99% in domain 3, with only a single amino acid difference. Similarly, just four amino acid differences in the set of five SRCR domains (99.2% identity) distinguish the human and *Rhesus macaque* sequences, the identity over the entire sequence of SSc5D being 94%.

In contrast to mammals, we have been unable to identify unequivocal orthologs for SSc5D in fish, birds or an amphibian (*Anoliscarolinensis*). Together with the high level of expression of transcripts in placenta detected by Northern blot analysis (Fig. 3) this suggested that the expression of SSc5D might be restricted to placental mammals. We

were, nevertheless, able to identify the probable ortholog of SSc5D in the genome of the marsupial *Monodelphis domestica* (laboratory opossum), using the programme BLAST and by taking into account the genomic organization within syntenic blocks of the human and opossum chromosomes (Fig. 11). However, the gene structure of the opossum ortholog is significantly different from that of human and rodent SSc5D, having only two scavenger-type domains (with 75% and 76% identity with human SSc5D domains 4 and 5, respectively), suggesting that divergent evolution between the marsupial and placental mammalian molecules has taken place.

Somewhat surprisingly, however, a very good case can be made for there being a homologue in the Platypus genome (*Ornithorhynchus anatinus*). We have identified a partial genomic sequence in a genomic contig encoding a putative protein with three SRCR domains sharing a very high degree of similarity with the N-terminal three SRCR domains of SSc5D, *i.e.* between 89% an 91% identity (Fig. 10). Moreover, adjacent to the Platypus gene in the 5' direction is another proposed gene for which the best match is an *N*-acetyltransferase 14, mirroring the arrangement in the human genome (the two Platypus genes are present on a contig yet to be incorporated into the genome, so analysis of genes in the 3' direction is not possible). The Platypus is an egg-laying monotreme believed to have diverged from other mammals before the separation of placental mammals and marsupials. Although we also found in a separate small contig a sequence coding for a scavenger domain with 67% and 76% identity with SSc5D domains 4 and 5, the putative Platypus protein still lacks a fifth SRCR domain and the mucin-like C-terminal extension present in the human protein, so it is unclear whether the two proteins are true functional homologues despite their very high sequence homology. The striking restriction in the expression of a five-domain form of SSc5D in monotremes, and the apparent loss of a protein with this structure in marsupials, along with the very high levels of expression of SSc5D transcripts in the placenta in humans, is suggestive of its having had some role in the evolution of placental function.

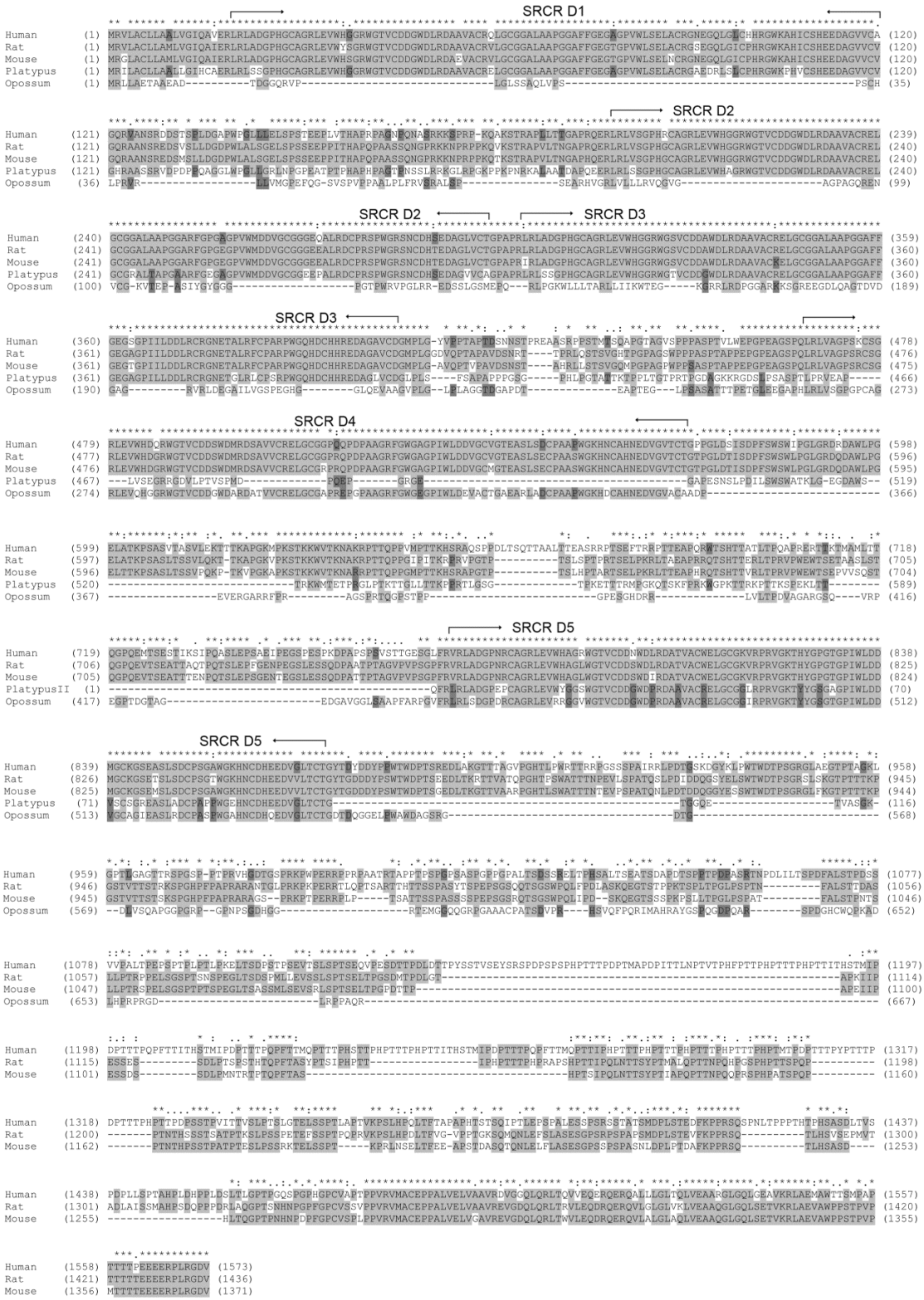

Figure 10 - Comparison of the predicted amino acid sequence of human SSc5D with mouse, rat and Platypus orthologous sequences. Identical residues shared between different sequences are grey shaded. Dark-grey shaded residues indicate different pair-wise identities between two sets of sequences. Identical residues in the human, rat and mouse sequences are denoted by asterisks; (+) indicates conserved amino acid substitutions, and (.) indicates semi-conserved substitutions. SRCR domain boundaries are indicated by arrows. NCBI reference sequences shown are: mouse SSc5D, NP 766596; rat SSc5D (hypothetical protein LOC308341), NP 001128017.1.; Platypus ortholog (hypothetical protein LOC100090938), NW 001709964.1.

The glycosylation-rich parts of the molecule are the least well conserved. The interdomain sequences between SRCR domains 1 and 2 (71%) and domains 4 and 5 (61%) still hold significant levels of identity in human and mouse sequences, but the most heavily glycosylated regions share much less homology, *i.e.* as low as 33% in the C-terminal mucin-like region. What has been conserved, however, is the likely very high level of glycosylation of the C-terminal part of mouse SSc5D, with a potential O-glycan likely to be added every 4 amino acids.

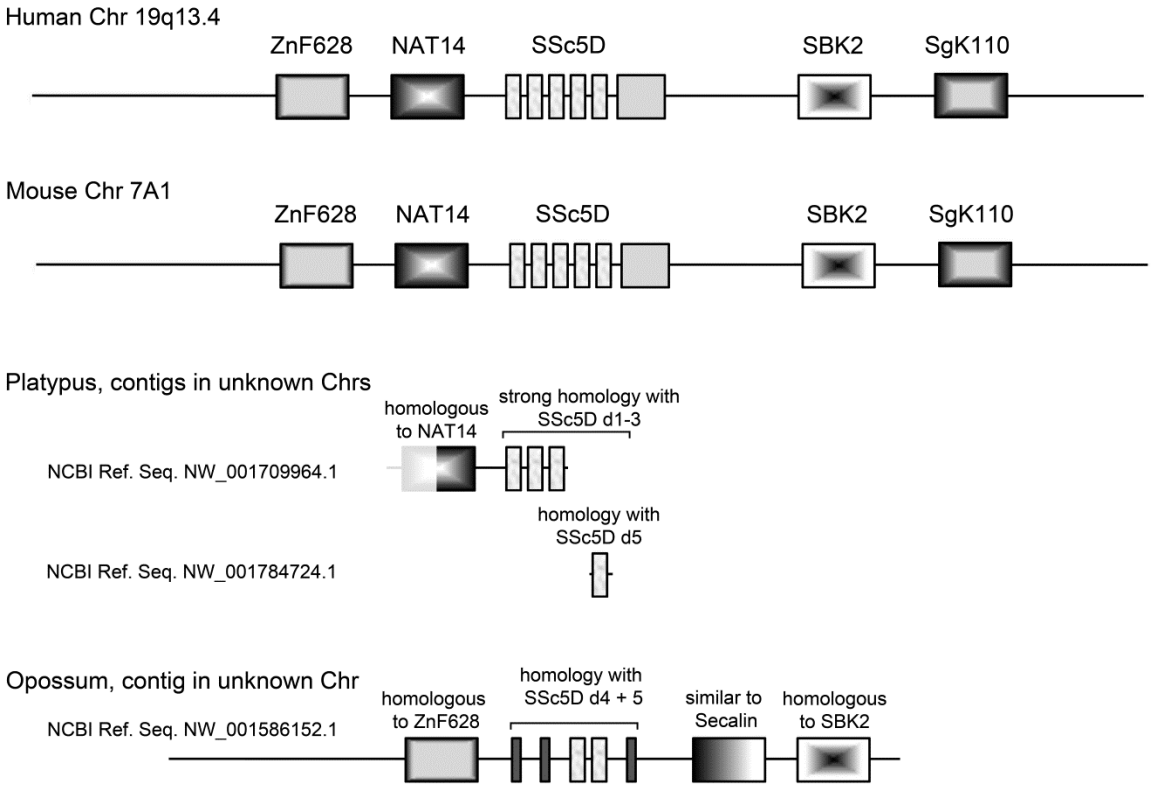

Figure 11 - Genomic organization of SSc5D-neighboring regions of the human, mouse, Platypus and opossum genomes. In human chromosome 19q13.4, the gene coding for SSc5D is flanked by the genes of *N*-acetyltransferase 14 (*NAT14*) and of the putative SH3-domain binding protein serine/threonine kinase SBK2 (Sugen kinase 069). Further afield are the genes of Zinc-finger protein 628 (ZnF628) upstream, and the putative serine/threonine Sugen kinase 110 (SgK110), downstream of SBK2. Mouse chromosome 7 presents a region (7A1) of conserved synteny, and as far as can be analyzed from available contigs, so does the Platypus genome: the first 3 SRCR domain-coding regions present in a genomic contig from the Platypus genome are very well conserved, and immediately 5' of the Platypus gene, and mirroring the arrangement in the human genome, there is a partial genomic sequence whose best match is human *N*-acetyltransferase 14 gene, with 66% identity over the last 107 amino acids of the translated sequence. A sequence containing strong homology with that of the fifth SRCR domain of human SSc5D was found in a separate small contig. The opossum genome appears to lack the gene coding for NAT14, and the gene structure of the ortholog of SSc5D has considerable differences, with only two regions of significant homology. However, the other genes in the opossum contig have homology with human genes in the vicinity of SSc5D: the amino acid sequence of human ZnF628 is 67% identical to the opossum ortholog, whereas SBK2 protein sequences have 70% identity over a 317 amino acid long stretch.

Evolution of the group B SRCR superfamily

Examination of the evolutionary relationships shared by the new, updated set of group B SRCR domains suggests a very complex history (Fig. 12). Identities among these domains vary from 20% to 80%. All five SRCR domains of SSc5D group together in a reconstructed phylogenetic tree of the full set of group B SRCR domains, strongly suggesting evolution *via* sequential intragenic duplication, as is clearly also the case for DMBT1. However, it is difficult to conclude whether the high degree of sequence similarity among these domains is a result of their recent evolution compared to other domains in the family or due to structural/functional constrains on the protein. The remarkable homology of the SSc5D domains among mammals, dating back to the divergence of egg- and non-egg-laying mammals, is indicative of profound functional constraints acting on at least some regions of these proteins. Domain 4 of S4D coclusters with the five SRCR domains of SSc5D, suggesting that an exon from a shared precursor of this gene may have seeded the generation of SSc5D at the chromosone 19q3 locus, or vice versa. The SRCR domains of CD5, CD6, Spα, M160, and CD163 exhibit very much more diverse relationships.

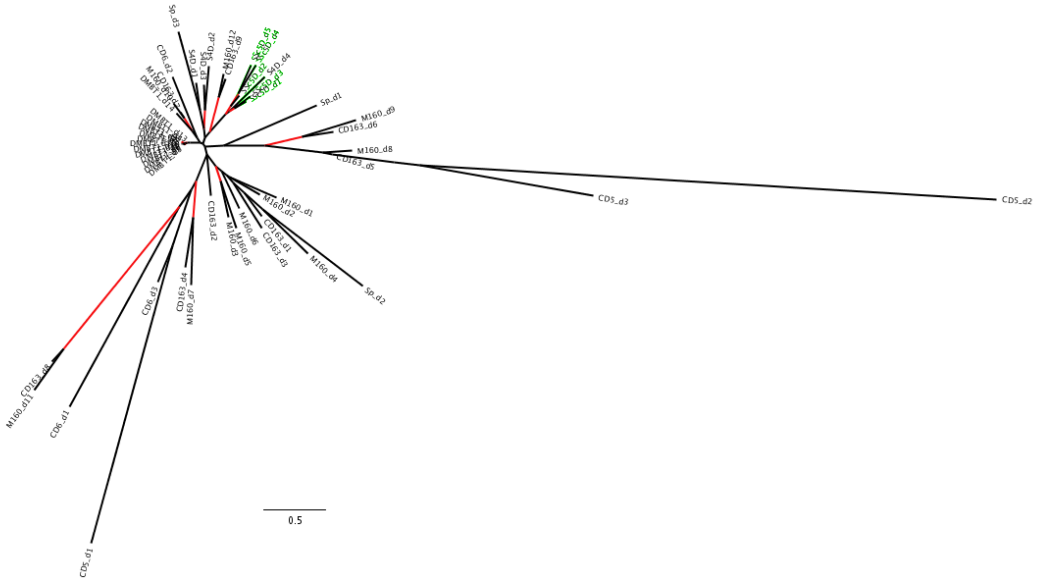

Figure 12 - Phylogenetic relationships of human SRCR domains. This un-rooted tree was reconstructed using the maximum likelihood method implemented in the PhyML program. Reliability for internal branches was assessed using the bootstrapping method (100 bootstrap replicates). Nodes occurring in more than 50 of 100 bootstrapped re-samplings are shown in red. The scale bar represents 0.5 expected amino acid replacements per site. The 5 Domains of SSc5D are shown in green.

Among these proteins, the SRCR domains exhibit greater inter- than intra-domain similarities but, with their low similarity, it is difficult to be certain whether the domains evolved through gene duplication and accumulated mutations that reduced sequence similarity, or through convergent evolution subsequent to domain shuffling. The similarities of domain 11 of M160 and domain 8 of CD163 are strongly suggestive of inter-protein domain shuffling.

Concluding remarks

The expression analysis of lymphoid cells revealed that SSc5D is primarily expressed in monocytes, and also in T-lymphocytes, and absent from B-lymphocytes. Unlike many proteins belonging to group B of the SRCR superfamily, SSc5D does not have a transmembrane region. The other soluble/secreted members are Spα and S4D-SRCRB; although in the listed NCBI sequences of DMBT1 there is no reference to a transmembrane segment, there is a 3' proximal exon that putatively encodes a membrane spanning sequence. The amino acid composition and domain organization of SSc5D most resembles that of S4D-SRCRB, which has no well characterized function, but is proposed to be involved in innate immunity (32). S4D-SRCRB is widely expressed and has four SRCR domains (32), whereas Spα is composed of three SRCR domains, is mainly expressed in macrophages and has anti-apoptotic properties (271, 306) SSc5D seems to have restricted expression, being exclusively expressed by monocytes/macrophages and T-lymphocytes. We speculate that, in addition to a possible role in placental function, SSc5D is active at the interface between innate and adaptive immunity.

Acknowledgments

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2

THE CYTOPLASMIC TAIL OF CD6 MEDIATES INHIBITION OF T CELL SIGNALING

Carine M. Gonçalves, Mafalda Pinto, Marta I. Oliveira, Alexandre M Carmo

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Introduction

One of the basic features of cells is the ability to respond to external stimuli and transform them into internal signals that lead to gene expression and phenotypic alterations. T cell activation is one of the processes that strongly depends on signal transduction. In the course of events there are several adapters, scaffold proteins, kinases and phosphatases, among others, that have a specific role in different pathways and are important factors that regulate the final outcome. CD6 is one of the players in the T cell activation process, but its true function remains a mystery to solve. CD6 is a 100-130 KDa membrane glycoprotein expressed in thymocytes and peripheral T lymphocytes (269). It belongs to the scavenger receptor cysteine-rich (SRCR) superfamily having three extracellular SRCR domains, a transmembrane region and a long cytoplasmic tail. Its function is still controversial, as some authors believe that CD6 has a co-stimulatory role in mature T cells (85, 307, 308), whereas there are some indications that, very much like the related receptor CD5, CD6 could have an inhibitory function in T cell signaling (75). CD5 and CD6 are thought to have evolved from the same ancestor gene and possibly they can share functions during lymphocyte development (309).

One of the most studied receptor-ligand interactions is that of CD6 binding to its ligand CD166 (310). CD166 is a widely expressed glycoprotein containing five Ig superfamily domains, of which the N-terminal domain has been shown to bind to the third, membrane-proximal, SRCR domain of CD6 (79, 310). The resulting lateral-type interaction is unusual, as most surface proteins bind “top-on-top” through N- or C-terminal domains. However, affinity and kinetic measurements revealed that, in solution, binding occurs with a *K*d of 0.4 –1.0 μM (311), relatively strong compared with most other leukocyte adhesion pairs, albeit in the range of low-affinity interactions characteristic of cell surface receptors. The CD6-CD166 interaction may have an important role during cellular maturation, activation and immune responses (74).

Binding of CD166 to CD6 induces the localization of CD6 in the immunological synapse, facilitating the tyrosine phosphorylation of CD6 upon TCR triggering. The long cytoplasmic tail of CD6 contains several tyrosine residues that can be phosphorylated upon T cell receptor (TCR) engagement and promote downstream signaling through SH2 domain-containing intracellular proteins (70). Yet, the exact mode how is CD6 phosphorylated remains unknown. Also, the intracellular domain contains potential proline-rich sequences where SH3 containing proteins can bind and signal through. The rat homologue of CD6, known as OX52, was shown to have the capacity to associate with

different protein tyrosine kinases, such as the Src-family kinases Lck and Fyn, the Tec kinase Itk and the Syk family kinase ZAP-70 (54).

Recent studies have shown that CD6 associates with SLP-76, a positive regulator of T cell activation (67), and with the scaffolding protein syntenin-1, possibly coupling CD6 to the cytoskeleton and other signaling effectors (68). CD6 has also been shown to associate at the surface of T cells with the structurally related receptor CD5 (54, 84), an inhibitor of T cell responses (18, 312, 313). CD5 and CD6 exhibit a shared pattern of expression during ontogeny, and the possibility of overlapping effects of these two proteins on repertoire selection has been previously suggested (309). Moreover, stimulation of rat T cells with anti-CD6 Abs enhanced the tyrosine phosphorylation of CD5 (54), suggesting that CD6 triggering may increase the inhibitory potential of CD5.

Here we examined the signaling properties of CD6 focusing on early and late signaling events. In marked contrast to what has previously been reported, we show that CD6 can be a negative modulator of T cell signaling and that this inhibitory effect is localized at the most C-terminal part of the cytoplasmic domain. However, the association of CD6 with the kinases Lck and Fyn was only observed using a cytoplasmic mutant that contains tyrosine residues (Y) 439 and 503. All these results suggested a different perspective about the CD6 function in T cells.

Materials and Methods

Cells and cell lines

Human PBMC from normal healthy adult volunteers were isolated by Lymphoprep (Nycomed Pharma) density gradient centrifugation. Isolation of T cells from PBMC was done using the RosetteSep Human T Cell Enrichment Cocktail (Stem cell Technologies) following the manufacturer's protocol. The E6.1 Jurkat cell line (314) was obtained from A. Weiss (University of California, San Francisco, CA). Cell lines were maintained in RPMI 1640, supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml) and streptomycin (50 mg/ml). Human embryonic kidney HEK-293T cells (315) were grown at 37°C in a 5% CO2 humidified incubator, in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5 mg/ml glucose, penicillin (100 U/ml), streptomycin (50 mg/ml) and 200 mM L-glutamine.

Constructs and transfections

E6.1 cells expressing cytoplasmic tail deletion mutants were obtained as follows: pHR-SIN-CD6-FL, -Cy5, -Cy37, -Cy70, -Cy135 and -Cy179 were amplified using the forward primer 5'TAGTAGGGCGCGCCCCAGCCTCTAGATGTGGCTCTTCTTC3', and reverses 5'CTACTAGGATCCCTATTATTTCCTTTAATTCTCAAGAGGATGAA3', 5' CTACTAGGATCCTTTGGGGATGGTGATG3',5'CTACTAGGATCCCTGGGCGCTGAAGT C3',5'CTACTAGGATCCCCTCGGGTGATACTGA3',5'CTACTAGGATCCCTCCAAGTTTG GGGG3' respectively.

For virus assembly, HEK-293T cells were transiently transfected with each of the pHR-SIN CD6 mutant constructs, together with pMD.G and p8.91 lentiviral vectors (316) in 6-well plates using Lipofectamine, according to the manufacturer's instructions. Supernatant was harvested at 48-72 h after transfection and centrifuged at 1,800 x g for 5 min at RT to remove cell debris. Virus-containing supernatant was added to $1x10^6$ E6.1 cells in 0.5 ml complete RPMI and let stand for an overnight. In the following day, 5 ml of RPMI medium were added to cells. Transfection efficiency was determined by cytometric analysis, as previously described (45), using a FACSCalibur (Becton Dickinson). For electroporation transfections, E6.1 cells or K562 cells (5×10^6) were transfected with 50 µg of hCD6 mutants or hCD166/pECFP-N2, respectively, and transferred into a Gene-Pulse cuvette (Bio-Rad, Hercules, CA). Electroporation was performed in a Bio-Rad Gene Pulser II electroporator (Hercules, CA) at 500 µF and 875 V. Cells were maintained in complete medium supplemented with 5 mg/ml of G418 (Invitrogen) at 37 ºC to select for positive cells.

Antibodies and reagents

Monoclonal antibodies used were: anti-phosphotyrosine mAb 4G10, HRP conjugated (Upstate Biotechnology); anti-CD6–MEM98 (EXBIO, Prague), anti-CD3-OKT3 (317); and the isotype control antibody IgG1 (BD Biosciences). Polyclonal Abs were: anti-Lck (DA3), anti-Fyn (BL90), anti-Itk and anti-ZAP-70, given by J. B. Bolen and M. G. Tomlinson (DNAX Research Institute, Palo Alto, CA); goat anti-mouse peroxidase conjugate (Molecular Probes); goat anti-rabbit peroxidase conjugate (Sigma-Aldrich); rabbit anti-mouse and rabbit anti mouse FITC-labeled from Dako and donkey anti-rabbit FITC-labeled from Jackson ImmunoResearch. ExtrAvidin peroxidase was purchased from Sigma-Aldrich and [γ-32P]ATP (>5000 Ci/mmol) was purchased from Amersham.

Pervanadate solution was obtained by adding 10 mM orthovanadate to hydrogen peroxide and completing the volume with water.

Calcium measurements

To measure total calcium release in human T lymphocyte populations or Jurkat cell lines expressing the different CD6 mutants, 3 x 10^6 cells were loaded with the calcium indicators Fluo-3 and Fura Red (Molecular Probes) at the final concentrations of 5 and 10 μM, respectively, in complete RPMI and incubated for 45 min at 37 ºC. Basal calcium levels were analyzed by flow cytometry for 1 min before addition of the agonist (OKT3 at 2 μg/ml), after samples were monitored for calcium release during 5 min. Analysis was performed using the FlowJo software (Treestar). The ratio FL1/FL3 was derived and plotted against time. Kinetic plots are represented as mean of the FL1/FL3 ratio.

CD6 knockdown using morpholino oligonucleotides (MO)

Knockdown of hCD6 from T lymphocytes was achieved through the transient transfection of CD6 MOs into T cells using Amaxa nucleofection. In brief, 1 x 10^7 cells were centrifuged, resuspended in 100 ml of pre-warmed nucleofector solution, mixed with 150 mM of MOs for CD6 (MO-CD6, 50-GTCTGGAGCTGTCTCTGGCTGCTAC-30, GeneTools), or 150 mM of scramble MO-control (MO-c; 50-GTgTGCAGCTCTCTCTGCCTGGTAC-30-fluorescein), and transferred into nucleofector cuvettes (Amaxa/Lonza). Nucleofection was performed in a Nucleofector II with the V-024 program. Cells were transferred to and maintained in complete RPMI 1640 medium at 37 ºC and used for analysis 72 h post-transfection. CD6 knockdown was confirmed by Western blotting, using MEM-98 for the detection of hCD6. Western blotting was performed as described below. The membranes were then stripped and restained with an Ab specific to β-actin. The densities of bands were measured with a Quantity-One version 4.6.9 imaging system (BioRad) and normalized with β-actin.

Cell surface biotinylation

For cell suface biotinylation, 2 x 10^7 cells were washed three times with ice-cold PBS and resuspended with 1 ml PBS containing EZ-LinkTM Sulfo-NHS-LC-Biotin (Pierce) at a final concentration of 0.5 mg/ml. After incubation for 10 min at RT, cells were thoroughly washed (four times with PBS) and lysed for 30 min at 4 °C in 1% Triton X-100

lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% (v/v) Igepal CA-630).

Immunoprecipitations and reprecipitations

Between 1 and 5 x 10^7 cells were lysed for 30 min on ice in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and 1% (v/v) NP-40 or TritonX-100). The nuclear pellet was removed by centrifugation at 11,000 x g for 10 min at 4 °C, and the supernatants were mixed with 150 ml of a 10% protein A Sepharose CL-4B (Amersham) slurry and with antibodies (1 – 10 µg) or antisera (1 – 3 µl). Samples were incubated for 90 min or over night at 4 °C. The beads containing the immune complexes were washed three times in 1 ml lysis buffer and either boiled for 5 min in SDS buffer for immunoblotting or washed for two more rounds in kinase assay buffer and subjected to *in vitro* kinase assays. For reprecipitations, the beads containing the immune complexes were boiled for 5 min in 2% SDS and diluted 8-fold with lysis buffer. After centrifugation, the supernatants were recovered and precleared for 30 min with 100 ml of protein A Sepharose beads (10% slurry). Proteins were incubated as mentioned earlier for 90 min. Immunoprecipitates were washed three times with 1 ml lysis buffer. Samples were boiled for 5 min in SDS buffer and subjected to SDS-PAGE.

Western blotting

Proteins were denatured in 2 x SDS buffer, separated by SDS-PAGE under nonreducing conditions and then transferred to HybondTM-C super membranes (Amersham) using i-Blot (Invitrogen). Membranes were blocked in 0.1% (v/v) TBST containing 5% (w/v) nonfat dried milk or BSA for 30 min, washed two times for 5 min with 0.1% TBST, probed with primary Ab (typically a 1:5,000 dilution) for 1 h at RT, washed one time for 15 min and then four times for 5 min with 0.1% TBST, and incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (1:20,000 dilution) for 1 h at RT. For detection of biotinylated cell surface antigens, membranes were incubated with ExtrAvidin peroxidase conjugate (Sigma). Membranes were washed again four times for 5 min with 0.1% TBST and subjected to detection. Immunoblots were developed using enhanced chemiluminescence – ECL Plus or ECL prime (Amersham) – and exposed to BioMax MR films (Kodak).

In vitro kinase assays

NP-40 assay buffer (30 µl) containing 10 mM $MnCl_2$, 1 mM Na_3VO_4, and 50 mCi (185 KBq) of [γ-32P]ATP was added to the beads containing the immune complexes, and *in vitro* kinase reactions were allowed to occur for 15 min at 25 °C. Reactions were stopped by the addition of 30 µl of 2 x SDS buffer, following which the samples were boiled for 5 min. Products were separated on SDS-PAGE gels, and autoradiography of the dried gels was done with BioMax MR films (Kodak).

Flow cytometry

For the detection of surface receptors, cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% NaN_3 (PBS/BSA/NaN_3), at a concentration of 5 x 10^6 cells/ml. Staining was performed by incubation of 1 x 10^6 cells/well with mAbs (20 µg/ml) for 15 min on ice, followed by rabbit anti-mouse FITC-labeled, in 96-well round-bottom plates (Greiner, Nürtingen). Data obtained by FACS were analyzed by FlowJo.

Proliferation assays and cytokine production

Proliferation assays were performed in 6-well cell culture plates. For T cell stimulation, K562 or K562-CD166 supplemented with superantigen (sAg) mixture (SEA, SEB and SEC3) were irradiated (6000 rad) and added to 6-well plates (2 x 10^4 cells/well). Human T cells (1 x 10^5 cells/well), prepared as described before, were labeled with 1 µM CFSE and co-cultured for 6/7 days. Cells were harvested and measured CFSE dilutions in FACS analysis using a FACSCalibur (Becton Dickinson). All proliferation assays were done in triplicates and means and SD are shown. For cytokine production analysis, E6.1 cells expressing the different hCD6 mutants were plated at 10^5 cells/well in complete RPMI, in the presence or absence of stimulation with PHA at 1 µg/ml. Supernatants were collected after 24 h and assayed in duplicate for IL-2 production by ELISA (Abcam) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software (Version 5.04 for Windows, Graphpad Software, La Jolla, CA, USA). Student's unpaired t-test was used to analyze continuous data; a w2-test for independence was used to analyze noncontinuous data

Results

Expression of human CD6 downregulates calcium signaling and IL-2 production

In the initial experiments we used primary human T cells. In order to obtain T cells devoid of CD6, we treated cells with hCD6 morpholinos (MOs) and obtained nearly 95% suppression of CD6 expression (Fig. 1A). Evaluation of calcium signaling by T cells, expressing or not CD6, was assessed following cell activation. Using 1 µg/ml of anti-CD3 mAb OKT3 to activate 3 x 10^6 cells/ml, it is possible to observe that OKT3 induced a marginal increase in calcium signals, whereas in CD6-depleted cells, calcium levels augment significantly (Fig. 1B).

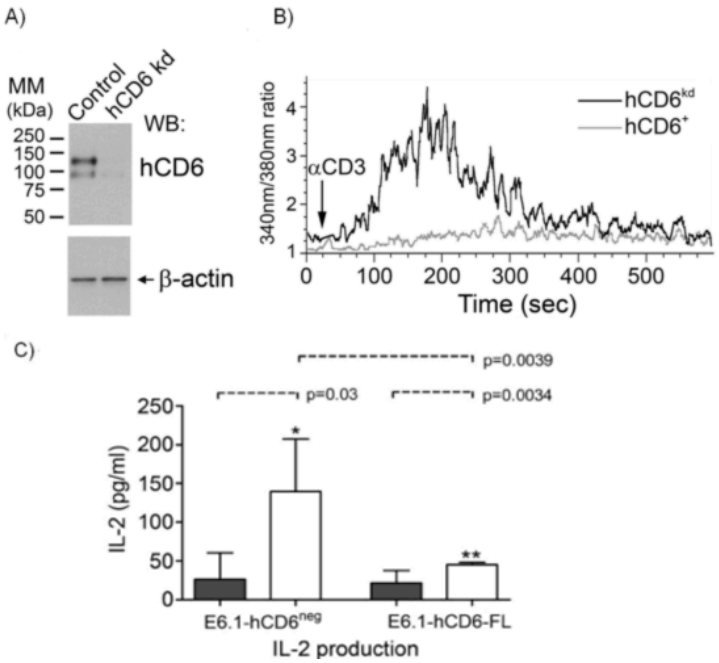

Figure 1 - CD6 attenuates early and late signaling events of T cell activation. (**A**) Purified human primary T lymphocytes were treated with specific hCD6 morpholino oligomers to suppress endogenous hCD6 expression ($hCD6^{kd}$), or with control scramble morpholinos (Control). Knockdown of human CD6 was evaluated by western blotting, using β-actin as a loading control. (**B**) Cells were incubated with calcium indicators and after stimulation with OKT3, evaluated for calcium mobilization by cytometry. Cells expressing hCD6 (grey line) display much lower calcium signals than cells where hCD6 was knocked-down ($hCD6^{kd}$, black line). **(C)** IL-2 production (pg/ml) by hCD6 negative ($hCD6^{neg}$) *versus* hCD6-expressing (hCD6-FL) E6.1 Jurkat cells, treated (white bars) or not treated (black bars) with OKT3. Data shown are mean +95% confidence intervals from three independent experiments. The probability that the production of IL-2 by $hCD6^{neg}$ and hCD6-FL E6.1 cells was similar, with and without anti-CD3 mAb stimulation, was assessed using Student's t-test (for *p*–values, see graph).

As expression of CD6 in T cells reduces early signals, we assessed whether this effect was also observed in downstream responses such as IL-2 production. We thus

measured IL-2 secretion in Jurkat cells expressing (E6.1-CD6-FL) or not (E6.1-CD6^{neg}) CD6, stimulated with OKT3 or with a negative control. Following stimulation, cells expressing CD6-FL produced three-fold less IL-2 than those devoid of CD6 (Fig. 1C).

The inhibitory role of CD6 is dependent of its cytoplasmic domain

We engineered an isoform of human CD6 lacking the cytoplasmic tail, termed CD6CY5, and that was then expressed in E6.1 cells previously sorted for the most negative CD6 population (E6.1-hCD6^{neg}), originating the E6.1-hCD6CY5 line (Fig. 2A).

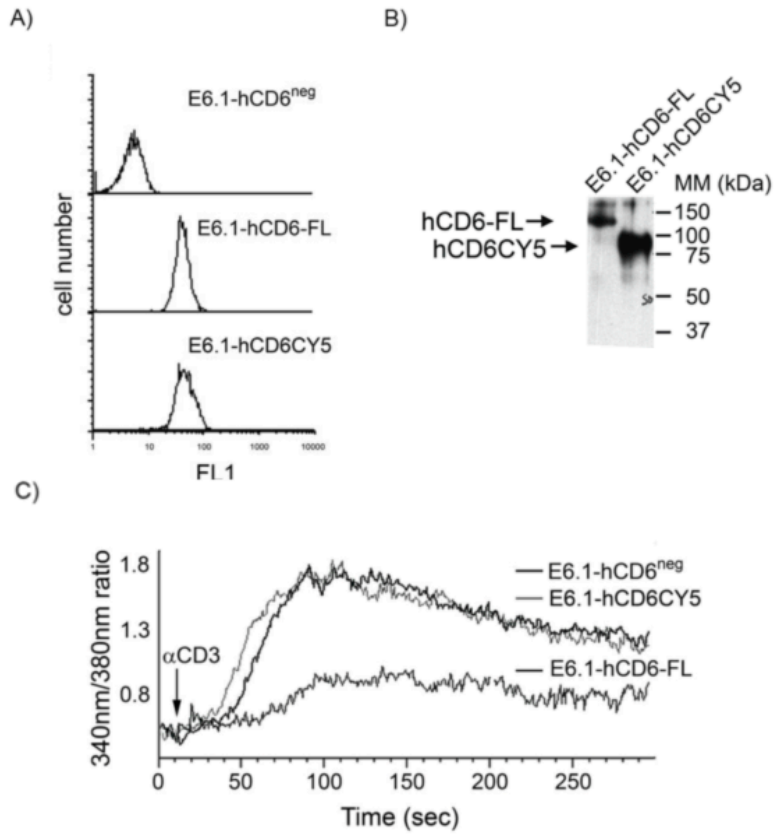

Figure 2 – CD6 cytoplasmic domain-dependence on signaling inhibition. **(A)** E6.1 Jurkat cells expressing no endogenous hCD6 (*top)*, full-length human CD6 (hCD6-FL, *middle*) or a cytoplasmic deletion mutant (hCD6CY5, *bottom*). **(B)** SDS-PAGE of surface biotinylated E6.1-hCD6-FL and E6.1-hCD6CY5 cells. **(C)** Calcium responses in E6.1 cells expressing hCD6-FL or hCD6CY5 and CD6-negative cells upon OKT3 stimulation. Measurements were similarly high in both CD6 negative cells and cells expressing CD6CY5, whereas cells expressing full length CD6 exhibited much poorer signaling capabilities.

E6.1-hCD6^{neg} cells were also stably reconstituted with full-length hCD6, to give E6.1-hCD6-FL. In each experiment, E6.1-hCD6-FL and E6.1-hCD6CY5 were FACS-

sorted so that the levels of hCD6-FL and hCD6CY5 were equivalent. We confirmed by immunoprecipitation of hCD6 from surface biotinylated cells and Western blotting, that E6.1-hCD6-FL cells expressed only the full-length hCD6 protein (~130 kDa) and that E6.1-hCD6CY5 cells expressed only the cytoplasmic domain-deletion form (~90 kDa; Fig. 2B). Calcium responses upon OKT3 stimulation (at 1 µg/ml using 3 x 10^6 cells/ml) were similarly high in both $hCD6^{neg}$ cells and cells expressing hCD6CY5, whereas cells expressing full-length hCD6 exhibited much poorer signaling capabilities (Fig. 2C), thus establishing an inhibitory role for the cytoplasmic domain regarding calcium signaling.

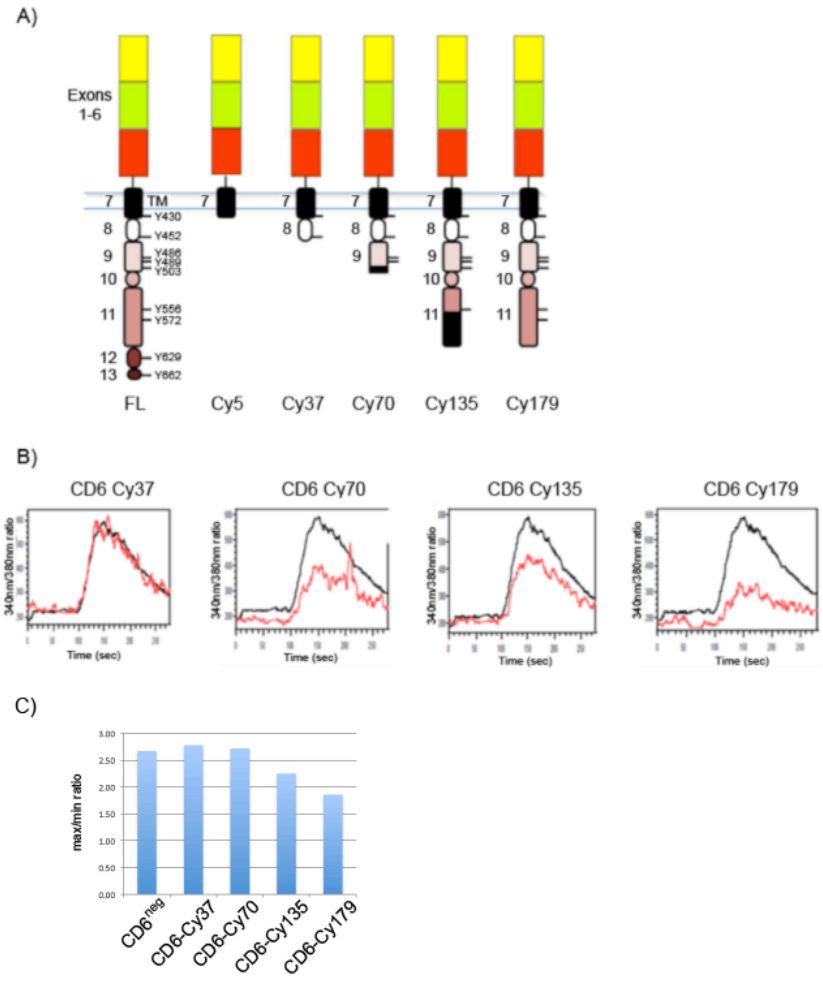

Figure 3 - Expression and signaling capacity of E6.1-CD6cyt mutants. **(A)** Schematic representation of cytoplasmic tail-truncated CD6 mutants constructed to map the inhibitory region. **(B)** E6.1-CD6cyt stable mutants obtained from electroporation were pre-incubated with Fluo-3, and upon CD3 stimulation, intracellular calcium levels were measured using cytometric analysis for 5 min. In red it is represented the calcium signals induced in the indicated mutant cells, and in black calcium fluxes induced in the control, CD6-negative untransfected E6.1 cells. Results represent one of at least three experiments having equivalent results. (**C)** Quantification of calcium signals of the experiment shown in (B), calculating the max/baseline ratio for each cell line.

To further characterize this inhibitory effect dependent on the cytoplasmic tail of CD6, and since this domain has several tyrosine residues that once phosphorylated can be targets for SH2 binding domains, CD6 constructs including/excluding different cytoplasmic sequences were constructed in order to map the regions that could be affecting signaling (Fig. 3A).

CD6 cDNA mutants were cloned into pcDNA3 and the vectors were then transfected by electroporation into E6.1 Jurkat cells. E6.1 cells expressing the different CD6 cytoplasmic mutants were activated and calcium measurements performed, which showed that the decrease in length of the cytoplasmic tail correlated with an increase in calcium signals (Fig. 3B and C). Cells with CD6 mutants with 70 or less amino acids in the cytoplasmic tail showed the same behavior as CD6 null cells, suggesting that the sites responsible for the inhibition of calcium signaling lie in the C terminal portion of the cytoplasmic region beyond residue 70.

One common problem associated with electroporation is the loss of protein expression after long periods of cell culturing. We then decided to make new stable cell lines using lentiviral transfection. We selected three mutants, CD6-Cy5, CD6-Cy70 and CD6-Cy179, clone them in a new vector, pHR-SIN, and induced their expression on E6.1 cells through lentiviral infection (Fig. 4A).

Following lentiviral transfection we analyzed their capacity to secrete IL-2 upon stimulation with PHA-P (Fig. 4B). With this approach it is possible to see that the biggest decrease in the IL-2 response is already observed in the mutant that has only 70 residues of the cytoplasmic tail, suggesting that the sequences responsible are localized between amino acids 5 and 70 of the cytoplasmic domain. So far, it was not possible to obtain the CD6-Cy37 mutant expressed in E6.1 cells using this transfection strategy. To confirm that these observations resulted from intrinsic effects on IL-2 production depending on the cytoplasmic sequences involved, and not due to behavioral differences between the two sets of cells, we again measured calcium fluxes of cells expressing the cytoplasmic deletion mutants upon CD3 triggering. Calcium responses are shown for CD6-Cy5, CD6-Cy70 and CD6-Cy179-expressing E6.1 cells, and again we can observe that the major drop in calcium release is observed in the mutant possessing more than 70 amino acids in the cytoplasmic domain (Fig. 4C and D).

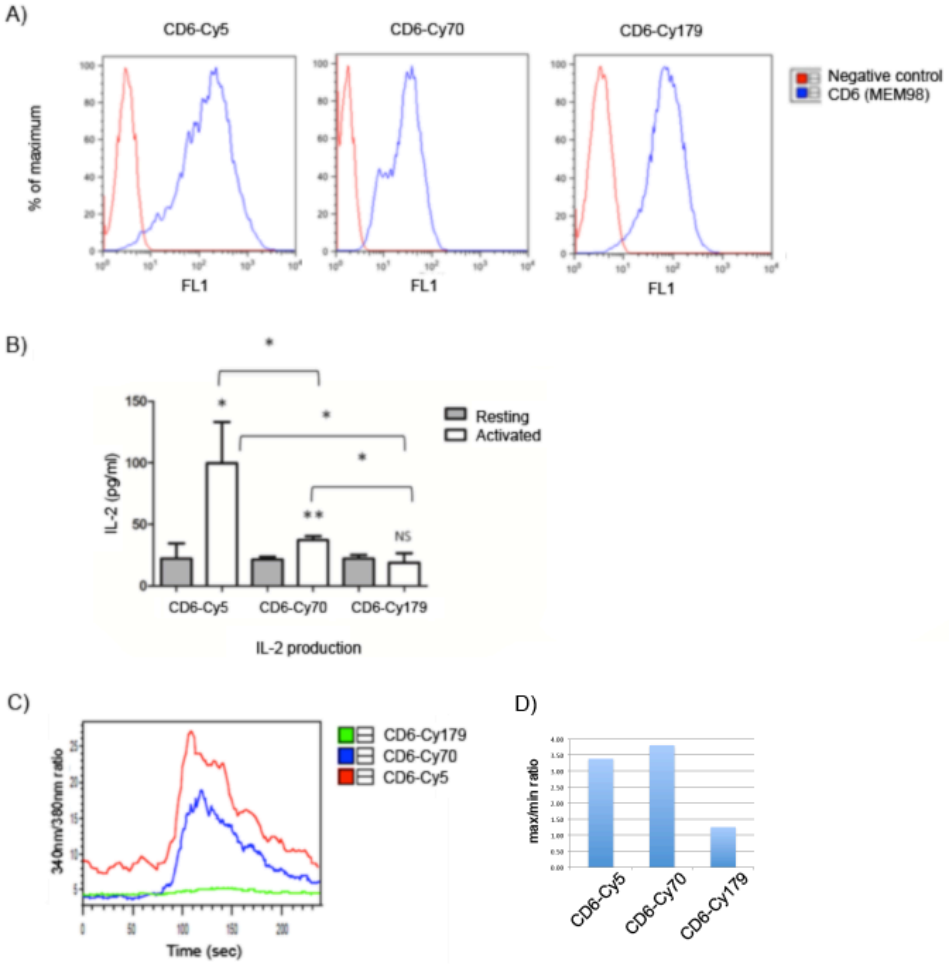

Figure 4 – Expression and signaling capacity of E6.1-CD6cyt mutants obtained following lentiral infection. **(A)** FACS analysis of the stable lines E6.1-CD6-Cy5, E6.1-CD6-Cy70 and E6.1-CD6-Cy179 using the CD6 mAb MEM98. **(B)** IL-2 production (pg/ml) with cells E6.1-CD6Cy5, Cy70 and Cy179 cells activated (white bars) or not (grey bars) with PHA (2µg/ml) for 24 h. Data shown represent the mean of 95% confidence intervals from three independent experiments. **(C)** Fluo 3 was incubated with E6.1-CD6Cy5, Cy70 and Cy179 for 30 min at 37 ºC and calcium release was detected by flow cytometry after activation with OKT3. (**D)** Quantification of calcium signals shown in (C), calculating the max/baseline ratio for each cell line.

The signaling differences of cells expressing CD6 cytoplasmic deletion mutants prompted us to evaluate the protein-tyrosine kinase activity within these mutants. Immunoprecipitated CD6cyt mutants were subjected to *in vitro* kinase assays. We were anticipating to detect similarities between the profile of phosphoproteins precipitated with CD6-Cy5 and CD6-Cy70 in case the CD6-binding proteins would have a role in regulating calcium fluxes, or between CD6-Cy70 and CD6-Cy179 precipitates if it was the case that associated proteins modulated IL-2 production. However, using the standard protocol of kinase assays we were not able to detect many differences between all three

immunoprecipitates, except for the presence of a phosphorylated protein of ~150 kDa associating only with CD6-Cy70 (Fig. 5A).

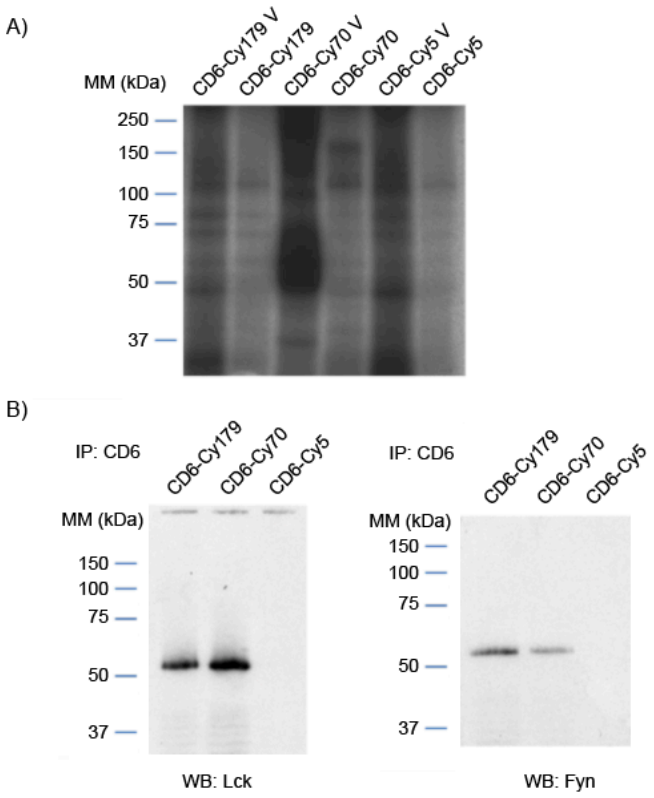

Figure 5 – Lck and Fyn associate with the cytoplasmic tail of CD6. **(B)** *In vitro* kinase assays of CD6 immunoprecipitates in E6.1 CD6 mutants treated or not with orthovanadate were performed in the presence of [γ-^{32}P]ATP. Phosphorylated products were separated by 10% SDS-PAGE and visualized by exposure of the dried gels to BioMax MR films. **(C)** The IPs from the previous experiment were also run in parallel gels and immunoblotted with Lck (on the right) and Fyn (left) polyclonal antibodies.

We then modified the assay, with the addition, during the kinase reaction, of sodium vanadate, a potent inhibitor of tyrosine phosphatases, keeping in mind that a phosphatase associating with CD6 might be influencing the inhibitory effect of the cytoplasmic domain. The use of vanadate permitted the visualization of a strongly phosphorylated protein of about 55 kDa, presumed to correspond to Lck and/or Fyn, only in association with the Cy70 deletion mutant (CD6-Cy70 V), but not with the Cy5 (CD6-Cy5 V) or Cy179 (CD6-Cy179 V) mutants (Fig. 5A). A first point is that Lck and Fyn only seem to associate with CD6 mutants having at least 70 amino acids of the cytoplasmic tail. This was confirmed by western blottings of CD6cyt mutant immune complexes using Lck and Fyn antibodies (Fig.

5B). The fact why Lck/Fyn are seen with CD6-Cy70 only when vanadate was used seems to indicate that a phosphatase that de-phosphorylates these kinases is also present in the CD6 immune complexes. The reason why the same behavior is not observed in the CD6-Cy179 mutants is more difficult to explain. A possibility is that a strong phosphatase, not inhibited by the concentrations of vanadate used, associates with the C-terminal part (between aa 70 and 179) of CD6. This implies that a mild, vanadate-sensitive, phosphatase activity is already present in association with the CD6-Cy70 mutant. Nevertheless, the fact remains that all three mutants display different profiles of associated kinase/phosphatase activities, which does not clarify whether the combination of the enzymes present has a role in the regulation of calcium fluxes or IL-2 production. Further dissection of the mechanisms and the players involved is required for a proper interpretation of the data.

CD6-CD166 interactions are not required for down-modulating Ca^{2+} signaling but important for cell proliferation

To address the role of the binding of CD6 to CD166 in the regulation of the signals modulated by CD6, we next focused in this intercellular interaction and its consequence in T cell proliferation. Preferentially we should use APCs that did not express CD166 and where we could transfect a CD166-expression vector. Unluckily, there are very few cells that can be used as APCs in functional assays and that lack CD166 expression. The erythroleukemic cell line K562 was an interesting candidate since CD166 is absent in these cells, however it lacks the appropriate MHC complexes used for activation with superantigens (sAg). Nevertheless, this cell line has been shown previously to respond, albeit modestly, to sAg stimulation. In order to promote the binding of CD6 to its ligand, and the consequent localization of CD6 at the immune synapse, K562 cells expressing CD166 (K562-CD166) were also used (Fig. 6A).

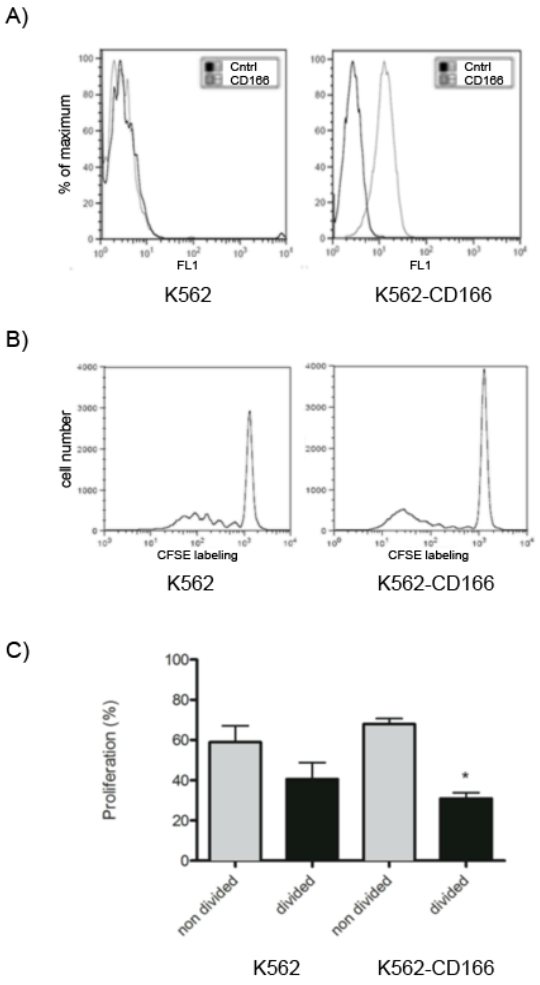

Figure 6 – The CD6-CD166 interaction decreases the proliferative behavior of the T cells. **(A**) Flow cytometry analysis of CD166 expression on K562 and K562-CD166cells. **(B)** Cell proliferation assays in conjugates formed between sAg-pulsed K562 cells or K562-CD166 cells and T cells. T cell interacting with CD166-expressing cells show less proliferation compared with T cells interacting with parental K562 cells. **(C)** Statistical analysis of T cell proliferation.

Human T lymphocytes isolated from blood were activated with sAg to promote synapse formation and incubated with K562 or K562-CD166 cells, used as APC. T cells were labeled with CFSE and were allowed to form conjugates with either K562 or K562-CD166 cells for six days. T cell proliferation was assessed by measuring CFSE dilution as cells divided. As seen in Fig. 6B and C, ligation of CD6 with CD166 induced the decrease of T cell proliferation, as APC cells expressing CD166 showed a reduced proliferative response. We concluded that the CD6-CD166 interaction constrains long-term signaling events in T cells.

Discussion

The importance of understanding the overall effect of receptors that have implications in T cell signaling is crucial to interpret how a proper immune response is initiated and maintained. This study shows for the first time that an important receptor expressed at the T cell surface, CD6, can downmodulate early and late T cell responses. Our main evidences are: (i) CD6 reduces Ca^{2+} responses in both human T cells and Jurkat cells, and the suppression of CD6 expression increases these responses; (ii) in addition to receptor proximal signaling, CD6 expression suppresses later events such as cytokine secretion; (iii) the cytoplasmic domain is responsible for these inhibitory effects; (iv) the ligand-dependent localization of CD6 to the synapse can have an effect in proliferation, as seen by CFSE uptake. These effects may be related to the association of CD6 with the kinases Lck and Fyn, and eventually with tyrosine phosphatases. We conclude from these experiments that CD6 has an important role in establishing thresholds for T cell activation.

CD6 was previously regarded to be a costimulatory receptor, still this assumption was largely built on ours and other group's reports in which CD6 was cross-linked together with the TCR using mAbs (54, 318, 319). An explanation for these observations is that since CD6 associates with protein tyrosine kinases such as Lck and Fyn, as seen in Fig. 5B and also with ZAP-70 (54), it could be expected that the mAb-induced coaggregation of CD6 with the TCR might promote the Lck/Fyn-mediated phosphorylation of CD3 ITAMs, leading to signaling. CD5 was originally proposed to be a costimulatory molecule for similar reasons (47, 320), but was subsequently shown to inhibit T cell activation and thymocyte selection (18).

Recent studies have shown that blocking the CD6–CD166 interaction with soluble recombinant ligands or mAbs reduces T cell activation and proliferation following the interaction of T cells with APCs (67, 85, 307, 311), supporting a costimulatory role for CD6. In these experiments, however, the blocking Abs and recombinant proteins used significantly diminished the number of T cell–APC conjugates that are formed, implying that the observed inhibitory effects were not simply due to the blockade of costimulatory processes. Zimmerman *et al.* (307) observed that a non-blocking CD6 mAb (M-T605) that targets domain 1 of CD6 rather than the ligand-binding domain 3, and therefore ought not to block the CD6/CD166 interaction, is a potent inhibitor of T cell proliferation. Almost complete inhibition of proliferation was also observed in cultures in which soluble CD166-Fc was used as a blocking agent, whereas the inhibition was much less pronounced when a CD6–Fc fusion protein was used. Similarly, Montero and colleagues showed that in the

cultures of T cells interacting with plate-bound recombinant CD166 and CD3 mAb, the inclusion of a nonblocking CD6 domain 1-binding Ab markedly reduced intracellular phosphorylation and strongly inhibited T cell proliferation (308). Given that only the reagents that interact directly with the CD6 molecule completely abolish T cell activation, it seems reasonable to propose that in these experiments, engaging CD6 delivers a negative signal to the T cells, as is now more exhaustively documented in the present experiments.

However, it is also possible that CD6 engagement strengthens T cell activation, albeit indirectly. The interaction of CD6 with CD166 is fairly strong (311), and is likely to increase cellular adhesion and in this way enhance T cell responses. Thus, a dual function for CD6 could be envisaged, whereby the balance of positive (stronger adhesion) and negative (inhibitory signaling) processes fine-tunes T cell activation. Hassan *et al.*, while showing that the interaction between CD6 and CD166 is important for T cell activation, have reported that an increase in the level of CD6 expression made T cells less responsive (67), in agreement with our present observations. The overall balance between inhibitory and activatory signals, including the strength of the signals initiated by the TCR, is likely to dictate the outcome of the activation process.

The molecular mechanisms through which CD6 constrains signaling are not yet determined, but it is possible that CD6 functions in a similar way of CD5, coupling with inhibitory tyrosine phosphatases, e.g. PP2 or SHP-1, or modulating the activity of signaling-enhancing tyrosine kinases (55, 321). Alternatively, given that CD6 associates with Lck and Fyn, it might sequester these kinases away from the TCR apparatus or it could function analogously to the IgSF glycoprotein CD2, which transduces mitogenic signals via its association with lipid rafts and the tyrosine kinases Lck and Fyn (186, 187), and inhibitory signals via its association with CD5 (45, 46, 322). Since CD5 and CD6 also associate at the surface of T cells and one major effect of CD6 stimulation is the phosphorylation of CD5 (54), it is feasible that CD5 can integrate or transform the signals generated by the triggering of CD6. CD6 full length is expressed in almost all T cells, however several studies showed the presence of CD6 intracellular isoforms produced at distinct stages of activation or differentiation (60, 323). As the cytoplasmic part of CD6 is highly tyrosine phosphorylated after T cell activation, the example of CD5 could be followed (312, 313), with a systematic and careful analysis of the various tyrosine residues present within the domain of CD6 undertaken. Since, as we show, the cytoplasmic domain is crucial for the inhibitory role of CD6, variation in its composition may translate into

different associations with effectors molecules that could very conceivably fine-tune activation thresholds in T cells.

Stable cell lines expressing CD6 cytoplasmic deletion mutants were analyzed in several approaches. In a kinase assay analysis, a higher degree of phosphorylation was observed in immune complexes of a CD6 mutant having only 70 amino acids of the cytoplasmic tail. This phosphorylation is probably due to the interaction of CD6 with Lck and Fyn. While it is possible that Lck and Fyn may have a role in the inhibition of CD6-signaling, by a still-undetermined mechanism (cells are more prone to activation with CD6 mutants that do not associate with either Lck or Fyn), a role for protein phosphatases is also likely. A possible association of a protein tyrosine phosphatase associating with the C-terminal part of CD6 could explain the reduced capacity of CD6 molecules having most of the cytoplasmic tail to induce calcium signals and IL-2 secretion. The differential combination of kinases and phosphatases associating with CD6 mutants having intermediate lengths possibly justifies the different profiles of calcium and IL-2 production elicited by cells expressing these mutants. Such results could indicate that the modulation of CD6 signaling is done at two different stages. However, further work is needed to examine the exact role that both Lck and Fyn, and possibly tyrosine phosphatases, have in their association with CD6, as well as to identify the residues that are implicated in these interactions.

The observation that, like CD5, CD6 also attenuates signaling in T and B cells stimulated via their physiological receptors, suggests that CD6 is not alone in its capacity to establish activation thresholds via its expression per se, and that this could be a general feature of inhibitory signaling proteins.

3

ANALYSIS OF LCK INTERACTIONS USING BIOLUMINESCENCE RESSONANCE ENERGY TRANSFER (BRET) AND LIPID RAFTS DISTRIBUTION

Carine M. Gonçalves, Raquel J. Nunes, John R. James, Marta I. Oliveira, Alexandre M Carmo, Simon J. Davis

includes results published in James JR *et al.*, Journal of Biological Chemistry, 286 (37): 31993-32001 (2011) and Nunes *et al.*, Journal of Immunology, 180:988-997 (2008)

Introduction

Upon TCR triggering, several signaling molecules are activated and contribute to different downstream events. An immune response is initiated when the TCR recognizes the antigenic peptide displayed by antigen-presenting cells (APCs). Communication between T cells and APCs is mediated by membrane surface receptors, and these interactions are then transmitted to the inside of the cells, many times involving changes in the distribution of the receptors and other signaling components within membrane microdomains. TCR signaling is achieved by a highly regulated process of membrane microdomain-based segregation of different molecules and this compartmentalization is essential to distribute, integrate and coordinate the spatio-temporal events after activation (324). Although many studies have been made to decipher this phenomenon, it is still unclear how membrane crosstalk is initiated and regulated.

Lateral segregation of plasma membrane (PM) into distinct microdomains commonly refers to lipid rafts (LR) (249). Lipid rafts are small and heterogeneous microdomains enriched mainly in sphingolipids and cholesterol. The importance of these microdomains is that proteins can be segregated and included or excluded from rafts, allowing for their diffusion (325) and facilitating or obstructing protein interactions within the plasma membrane (326). In resting cells, some proteins are considered to be raft resident, such as linker of activated T cells (LAT) (327), phosphoprotein associated with glycosphingolipid-enriched microdomain (PAG) (212), and Src kinases (328). Lipid modification of proteins, such as palmitoylation and myristoylation, contributes to the targeting of proteins to the rafts. Palmitoylation can occur in membrane-proximal cysteine residues within a conserved motif that also carries hydrophobic residues, CVRC in LAT and GCVC in Lck and Fyn.

One of the first events after T cell and APC engagement is the recruitment of Lck that phosphorylates the immune-tyrosine activation motifs (ITAMs) present in TCRζ and CD3 chains and the subsequent recruitment of the tyrosine kinase, ZAP-70 (329). One relevant question regarding the mechanism of TCR triggering and ITAM phosphorylation is the role of the CD4 and CD8 co-receptors, and the stoichiometry of interactions of these molecules with the kinase Lck. Whereas CD8 is a dimer, contradictory results have not established whether CD4 can dimerize, and therefore a possible functional dimerized co-receptor could have twice as much associated kinase activity.

Also, additional receptors that associate with Lck may have a role in helping directing the kinase to the sites of signaling initiation. One such important T cell accessory receptor that also facilitates the adhesion between T cells and APCs by adjusting the

optimal intercellular membrane spacing is the glycoprotein CD2. CD2 binds to CD58 in humans or CD48 in rodents, which are expressed on the surface of the APC. Concomitantly with its function as an adhesion molecule, CD2 has a role in the process of signal transduction. CD2 interacts with the inhibitory receptor CD5 (45, 46), and through this association is able to strengthen modulatory signals at the T cell surface (45, 322). Association of CD2 with both Lck and Fyn was also demonstrated and through these kinases CD2 can positively contribute to downstream signaling pathways (186, 189, 330). CD2 can also translocate to lipid rafts upon mAb crosslinking or following association with CD58 during conjugate formation (188).

Molecular and biochemical approaches have been taken to study protein interactions and signaling within T cells. One important method used to investigate the dynamics of interactions is Bioluminescence Resonance Energy Transfer (BRET) (331). This emerging methodology directly monitors protein interactions *in situ*. It is also a useful tool for characterizing the quaternary structures of cell surface molecules. BRET relies on nonradiative energy transfer between donor and acceptor fluorophores (332). If an appropriate acceptor is in close enough range from the other molecule, within a distance between 10 and 100 Å, it will be excited to a higher energetic state and energy transfer will occur, emitting photons with longer wavelengths. An optimized version of this technology, the $BRET^2$ assay, uses DeepBlueC, a modified form of the natural substrate, which has maximal emission, and GFP^2, a UV-GFP variant, which when excited provides a greater signal resolution (333). Resonance energy transfer techniques, such as fluorescence resonance energy transfer (FRET) and BRET are important methods that allow the measuring of constitutive and dynamic protein-protein interactions and the activity of several signaling pathways. The main advantage of BRET over FRET is that BRET, as it does not need an external light source, avoids problems associated with autofluorescence, photobleaching and coincident excitation of both donor and acceptor fluorophores, frequently formed with FRET. The great sensitivity of BRET (334) allows the detection of weak interactions at physiological levels of expression. Nevertheless, as will be seen, substantial energy transfer arises from random interactions. Here, we examine the organization of CD4, CD2, Lck and Fyn and study their molecular interactions within T cells and how these interactions can regulate T cell signaling.

Material and Methods

Cells and Antibodies

All cell lines were grown in 37 ºC incubators under 5% CO_2 conditions. HEK-293T, transformed human endothelial kidney (HEK) 293 cell line (315), was used for all transient expression BRET studies since cells are adherent, highly transfectable and offer good expression of almost all mammalian genes using the CMV promoter. HEK-293T cells were grown in DMEM (Sigma) supplemented with 10% FBS (Sigma), 2 mM glutamine (Sigma), and antibiotics (Sigma) and passaged using trypsin (Sigma). The Jurkat T cell line E6.1 (335) was derived from the peripheral blood of a 14 year old boy suffering from leukemia, and obtained from A. Weiss (UCSF, CA). E6.1 cells expressing full-length rat CD2, E6.1-CD2 (CY whole) (referred to in this text as E6.1-CD2) (336), or expressing CD2 cytoplasmic deletion mutants, E6.1-CD2(CY 6), (CY 40), (CY 66), (CY 81), and (CY 97), which possess respectively the first 6, 40, 66, 81, and 97 amino acids of the cytoplasmic tail (336, 337), were provided by M. Puklavec (University of Oxford, U.K.). E6.1-CD2 (CyΔ7-40) has a deletion from aa 7 to aa 40 of the cytoplasmic tail. All Jurkat cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 1mM sodium pyruvate (Invitrogen) and antibiotics.

Monoclonal Abs recognizing rat CD2 were OX-54, OX-55 (338), and OX-34 (339). The anti-phosphotyrosine mAb 4G10 HRP-conjugated was from Upstate Biotechnology. Polyclonal Abs and conjugates were: rabbit anti-LAT, from Upstate Biotechnology; rabbit anti-Lck, raised against a peptide of aa 39–64 of murine Lck, a gift from J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands); BL90, polyclonal anti-Fyn, a gift from J. Bolen and M. Tomlinson (DNAX Research Institute, Palo Alto, CA); goat anti-mouse peroxidase conjugate, purchased from Molecular Probes; and goat anti-rabbit peroxidase conjugate, from Zymed Laboratories.

Western blotting

Proteins were separated by SDS-PAGE in non-reducing conditions and then transferred to Hybond-C-extra membranes by electroblotting. Membranes were blocked in TBS, 0.1% (v/v) Tween 20 (TBS-T), containing 5% (w/v) non-fat dried milk, probed with unconjugated primary antibody for 1 h and revealed with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (1:20,000 dilution). Immunoblots were developed using ECL–plus reagents (Amersham Biosciences) and exposed to CL-XPosure films (Pierce).

cDNA cloning, plasmids and transfections

Jurkat E6.1 cells expressing CD2 with a deleted sequence between aa 7 and 40 of the cytoplasmic tail (rat CD2 (Δ7–40)), were produced as follows: pKG5-CD2–(Δ7–40) vector were obtained by PCR amplification using pRCD2–11 (336) as template, with the forward primer 5'-CTGCAAGAGGAAAAAACGGAAC/CCAGTGGCTTCCCAAGCT-3' and a reverse primer with the complementary sequence. The amplified product encodes a CD2 sequence in which the codon of aa 6 of the cytoplasmic tail is followed (separated by a slash) by the codon of aa 41, thus excluding aa 7–40. The resulting amino acid sequence at the junction is KRKKRN/NPVASQ. The PCR product was treated with *Dpn*I to digest the parental cDNA without the deletion, and used to transform competent cells.

A chimeric pKG5-CD2/PAG/CD2 was produced (GenScript), in which the sequences coding for the transmembrane region and six amino acids of the cytoplasmic domain of rat CD2 were substituted by the corresponding sequences encoding the transmembrane region and nine amino acids of the cytoplasmic tail of PAG, including the palmitoylation sequence. The sites of junctions of the chimeric protein are shown by slashes: PEKGLP/LWGSLA. . . .CDREKK/RRKGEE. The obtained chimeric cDNA was subcloned into the *Bam*HI restriction site of the expression vector pKG5. For simplicity, the mutant molecule is referred to in this study as CD2/PAG.

The Plasmid pKG5-CD2 (Δ7–40) was used for transfecting E6.1 cells. Briefly, cells at 1 x 10^7 cells/ml in ice-cold PBS were put into 0.4-cm electroporation cuvettes, mixed at 4 °C with plasmid at a final concentration of 0.1 mg/ml, and pulsed with 0.62 kV and 25 μF using a Gene Pulser II electroporator from Bio-Rad. Cells were transferred to culture flasks containing RPMI 1640 with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G (50 U/ml), and streptomycin (50 μg/ml), all from Invitrogen Life Technologies. After 48 h, the selection antibiotic (G418 at 1 mg/ml) was added to the cultures. Following selection, cells were analyzed for expression by cytometry and immunoblotting.

The Lck-deficient J.CaM1 cell line (340) was transfected with pRCD2–11 or pKG5-CD2/PAG/CD2, and G418-resistant cells that expressed rat CD2 were selected and named J.CaM1-CD2 and J.CaM1-CD2/PAG, respectively. To generate J.CaM1/Lck(KD)-CD2 cells, the newly obtained J.CaM1-CD2 cells were cotransfected with pSRα-Lck-KD, a plasmid containing Lck cDNA carrying a mutation at the ATP binding site (K273A) (194). J.CaM1 cells reconstituted with wild-type Lck, J.CaM1/Lck(WT), and with a Lck molecule having Cys3 mutated to Ala3, J.CaM1/Lck(C3A) (341) were obtained from P. Kabouridis (Queen Mary, University of London, London U.K.), and transfected with pRCD2–11, to handle J.CaM1/Lck(WT)-CD2 and J.CaM1/ Lck(C3A)-CD2 cells, respectively.

For BRET experiments, the complete sequences of all genes and chimeras, inclusive of their native signal peptide sequences, were amplified by PCR from cDNA or full length plasmids and cloned in-frame into either pGFP2-N3 or prLuc-N3 (PerkinElmer), mutating the stop codon to an appropriate restriction site using the oligonucleotide pairs listed in Table 1. All constructs were sequenced to check reading frame and integrity. Transient transfection of HEK-293T was performed using GeneJuice (Novagen) according to the manufacturer's protocol. Briefly, six-well plates were seeded with 6 x 10^5 293T cells to give 80% confluence on the day of transfection. Constructs were co-transfected in each well as "BRET pairs", resulting in the expression of single proteins as both Luc and GFP fusion, or of two distinct proteins in the form of Luc and GFP fusions. Transfection was performed using 1 µg of total DNA, with the ratio of GFP:Luc constructs ranging from 1:2 to 66:1, since this gave the most usable data. This was achieved by keeping the volume and concentration of the DNA at 20 µl and 0.05 µg/ml, respectively. For the 19:1 ratio of the Lck BRET pair, for example, 19 µl of LckGFP DNA at 0.05 µg/ml and 1 µl of LckLuc DNA at 0.05 µg/ml were co-transfected. In all experiments, the sGFP-Luc construct was also transfected in a separate well and used as a positive control.

Flow cytometry

Cells were washed and resuspended in PBS containing 0.2% BSA and 0.1% NaN_3 (PBS/BSA/NaN_3), at a concentration of 1 x 10^6 cells/ml. Staining was performed by incubation of 5 x 10^5 cells/well with mAbs (20 µg/ml) for 15 min on ice, in 96-well round-bottom plates (Greiner). Flow cytometry analysis was performed in FACS Calibur (BD Biosciences) using Cell Quest.

Table I – Primers used to amplify the different constructs for BRET analysis

Construct		Oligonucleotides (5'->3')	Restrictions	F/R
CD4	1	TAGTAGCTGCAGGGCAAGGCCACAATGAACC	*Pst*I	FWD
	2	CTACTAGGATCCATGCGTCTCTGATTCAGGCC	*Bam*HI	REV
CD4^{TM}	3	CTACTAGGATCCCTTCGGTGCCGGCACCTGACAC	*Bam*HI	REV
CD4^{Ex}	4	TAGTAGACGCGTCTGCAGGGCAAGGCC	*Mlu*I	FWD
	5	CTACTAGGATCCCTTCGGTGCCGGCACCTGACAC	*Bam*HI	REV
Lck	6	TAGTAGACGCGTCTCAAGCAGGCCACCATGGGCTGTGGCTGCA GCTCAC	*Mlu*I	FWD
	7	CTACTAAAGCTTAGGCTGAGGCTGGTACTGGC	*Hind*III	REV
Fyn	8	TAGTAGACGCGTCTCAAGCAGGCCACCATGGGCTGTGTGCAAT GTAAGG	*Mlu*I	FWD
	9	CTACTAAAGCTTCAGGTTTTCACCAGGTTGGTAC	*Hind*III	REV
CTLA4	10	TAGTAGAAGCTTGCCACCATGGCTTGCCTTGGATTTCAG	*Hind*III	FWD
	11	CTACTAGGTACCTTGATGGGAATAAAATAAGGCTGAAATTGC	*Kpn*I	REV
CD86	12	TAGTAGGATATCGACTCTCCCCATGCGCTCAAGC	*Eco*RV	FWD
	13	CTACTACCGCGGAAAACATGTATCACTTTTGTCGCATGAAG	*Sac*II	REV
CTLA4^{TM}Fyn	14	GCTTTCTCCTCACAGCTGTTATGGGCTGTGTGCAATGTAAG	—	FWD
	15	CTTACATTGCACACAGCCCATAACAGCTGTGAGGAG AAAGC	—	REV
Lck$^{C20/23}$	16	GAAAACATCGATGTGTCTGAGAACTCCCATTATCCCATAGTC	—	FWD
	17	GACTATGGGATAATGGGAGTTCTCAGACACATCGATGTTTTC	—	REV
Lck^{S6}	18	TAGTAGGGTACCAGCTCACACCCGGAAGATGAC	*Kpn*I	FWD
		PRIMER 7		REV
Nck	19	TAGTAGACGCGTGCCACCATGGGCTGTGGCTGCAGCTCACACA TGGCAGAAGAAGTGGTGG	—	FWD
	20	CTACTAGGATCCGATAAATGCTTGACAAGATATAA	—	REV
PD1-zeta	21	TAGTAGCCGCGGCTCCAGGCATGCAGATCCC	*Sac*II	FWD
	22	CTACTAGGATCCACGTCTCTTGTCCAAAACATC	*Bam*HI	REV
CD2^{Ex}Lck	23	TAGTAGCTGCAGCCCGTAAGATGAGCTTTCC	*Pst*I	FWD
	24	CTACTAGGTACCCCTTTTGGTGATATAGAAAACGAGCAG	*Kpn*I	REV
CD4^{Ex}Lck	25	TAGTAGAAGCTTGGCAAGGCCACAATGAACC	*Hind*III	FWD
	26	CTACTAGGTACCGACACAGAAGAAGATGCCTAGCCC	*Kpn*I	REV
	27	TAGTAGGGTACCATGGGCTGTGGATGCAGC	*Kpn*I	FWD
	28	CTACTAGGTACCAGGCTGAGGCTGGTACTGGC	*Kpn*I	
CTLA4^{TM}Lck	29	CTACTACGCGTAACAGCTGTGAGGAGAAAGC	*Mlu*I	REV
	30	CTACTACGCGCGAGGCTGAGGCTGGTACTGGC	*Mlu*I	REV
CD4^{FL}Lck^{FL}		PRIMER 1		FWD
		PRIMER7		REV
CD4^{TM}Lck$^{C20/23}$		PRIMER1		FWD
		PRIMER18		REV
CD6^{FL}	31	TAGTAGAGATCTGCTCCAGACATGTGGCTCTTCTTCGGGATC	*Bgl*II	FWD
	32			REV

Capping and immunofluorescence microscopy

All procedures were performed at 4 °C unless otherwise described. Cells were washed in RPMI 1640, resuspended at 2 x 10^6 cells/ml in RPMI 1640/5% FCS, and incubated for 10 min with the mAb OX-34-FITC conjugate (Caltag Laboratories) at 5 µg/ml. Cells were washed twice with PBS containing 0.2% BSA, resuspended in RPMI 1640/5% FCS, and incubated for 15 min at 37 °C to allow for Ab-induced capping. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and then incubated with saponin (0.4% w/v) for 20 min. Anti-LAT mAb (20 µg/ml) was added for 10 min to the preparations, followed by a PBS wash and incubation for 10 min with polyclonal goat anti-rabbit Alexa Fluor 568 conjugate (Molecular Probes) at 10 µg/ml, and again washed. In parallel, cells without CD2 cross-linking were fixed, permeabilized, and posteriorly labeled with OX-34-FITC and anti-LAT and goat anti-rabbit Alexa Fluor 568. Cells were plated onto glass coverslips and mounted in Vectashield medium (Vector Laboratories). Stained preparations were observed with an AxioImager Z1 microscope (Carl Zeiss), and images acquired with Axiocam MR v.3.0 camera (Carl Zeiss). Images were processed with Photoshop 6.0 (Adobe Systems). The percentage of colocalization represents the counts of CD2 caps that also colocalize with LAT, quantified with blind scoring, counting a minimum of 200 caps in each of two experiments. Each experiment was observed by two independent examiners.

Bioluminescence Resonance Energy Transfer (BRET Assay)

The BRET used was type I assay, consisting in the variation of acceptor-donor ratio. Cells were harvested from wells 24 h post-transfection using cold PBS, pelleted at 600 x g for 3 min in a microcentrifuge and resuspended at ~1.5 x 10^6 cells/ml in minimal essential medium (Gibco). For each transfection, 10 µM DeepBlueC (final concentration) was added to 100 µl cells in a 96 well OptiPlate. Light emission in the 410±40nm (RLU-*A*) and 515±15nm (RLU-*B*) wavelength ranges was collected on a Fusion microplate analyzer (PerkinElmer). Background values were taken from cells that were untransfected but otherwise treated in an analogous manner. The positive control (sGFP-Luc) that consists of GFP[2] genetically fused to Luc was analyzed similarly. To determine GFP and Luc expression, 100 µl cells were dispensed in a separate well, before exciting at 425±10nm and measuring emission at 515±15nm. The same well was then incubated with 10 µM coelenterazine-*h* for 2 min before reading again.

Results

In the previous chapter we have shown the capacity of CD6 to associate via its cytoplasmic tail with the protein kinases Lck and Fyn. We wanted to further pursue the study of these interactions using BRET. Furthermore, we were also interested in finding out whether CD6 could have the capacity to self-associate at the cell membrane and whether this feature could correlate or contribute to its inhibitory properties. We had demonstrated that its relative CD5, also an inhibitor of T cell signaling, was able to form loose dimers at the cell surface (55). Therefore, to take advantage of our expertise in the use of this powerful technique to analyze protein stoichiometry and interactions, we made constructs to fuse full length CD6 (CD6-FL), as well as a mutant devoid of the cytoplasmic tail (CD6-TM), with both GFP and Rluc to perform the BRET analysis. These proteins were to be used as BRET pairs to assess dimerization and to investigate the association with Lck and Fyn, constructing the equivalent Lck and Fyn fusion proteins. Unexpectly, when we plotted $BRET_{eff}$ over the ratio between donor and acceptor, the values obtained were higher than 1 for CD6-TM and almost 0 for CD6-FL (Fig. 1A).

These and many other subsequent assays with disappointing results meant that probably the proteins were not being folded correctly or they were retained in the ER. To obtain confirmation, we observed the expression and localization of the proteins by fluorescence microscopy and found that most of the protein was retained in ER and that the expression rate for CD6-FL was very low compared with that of CD6-TM (Fig. 1B). Although we used different expression strategies and different constructs, we realized that it was not feasible to perform BRET analyses on CD6. We had to turn our focus to a slight different direction, but nevertheless decided to proceed with the analysis of kinase dimerization and of the stoichiometry of interactions between Lck and Fyn with other relevant signaling effectors.

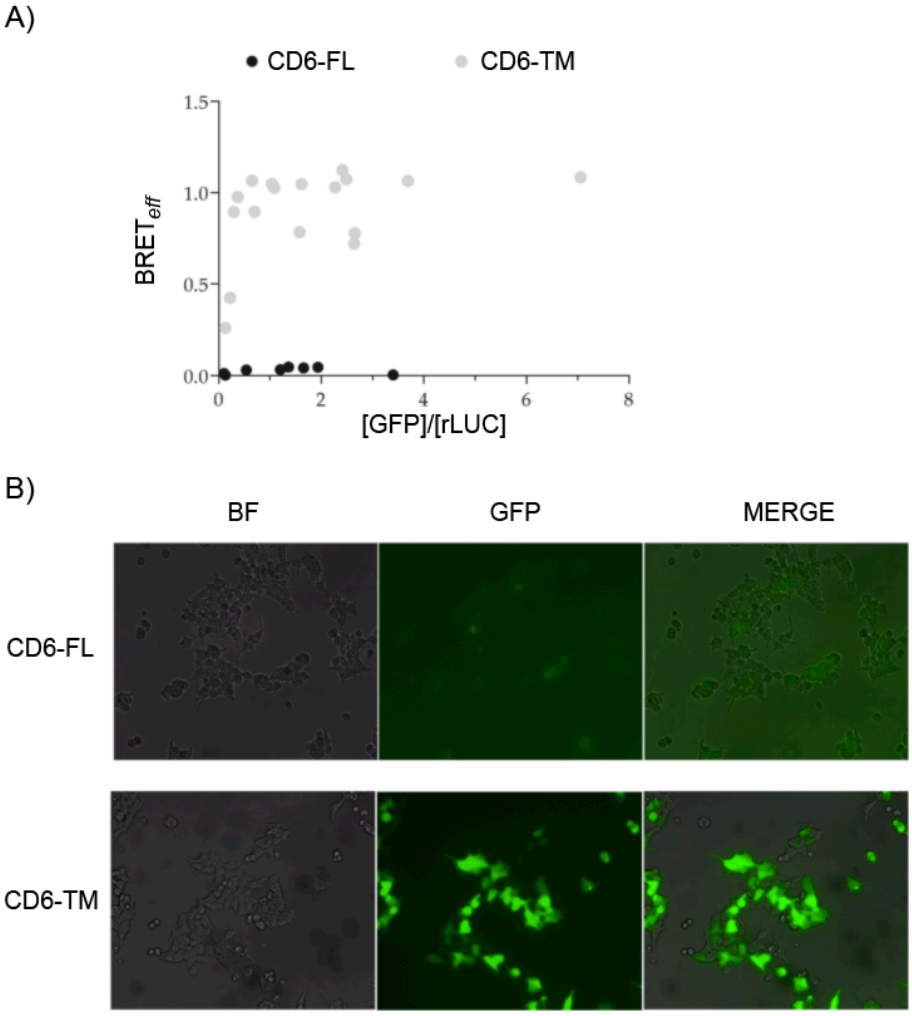

Figure 1 – BRET analysis of CD6. **(A)** 293T cells were transfected with both CD6-FL and CD6-TM for 18 h prior to BRET evaluation. BRET$_{eff}$ for CD6-FL was almost null whereas for CD6-TM was higher than 1. Both values were not acceptable for a proper BRET analysis, meaning that the analysis of CD6 through this approach was not possible. **(B)** Microscopy analysis of transfected 293T cells with both CD6 forms show that CD6-FL expression is very low compared with CD6-TM. Nevertheless both CD6 forms are retained in ER and virtually no expression at cell surface is observed.

BRET analysis of Lck and CD4 self-associations

The full length protein-encoding sequences of CD4 and Lck were genetically fused to either Luc or GFP and the resulting expression vectors were transfected into 293T cells, as previously described (259). Initially, we assessed the capacity of CD4 and Lck to dimerize independently of one another. BRET$_{eff}$ values measured for CD4 were high and indicative of oligomeric interactions (342), while Lck showed very low BRET$_{eff}$ values, even smaller than those detected for monomers (Fig. 2A and B). Previous work had established that the Bret$_{eff}$ values obtained for CTLA-4, CD80 and CD86 can be

representative of interactions of constitutive dimers, weak dimers and monomers, respectively (342). The values obtained are indicated in Fig. 2C and serve to categorize both CD4 and Lck BRET pairs.

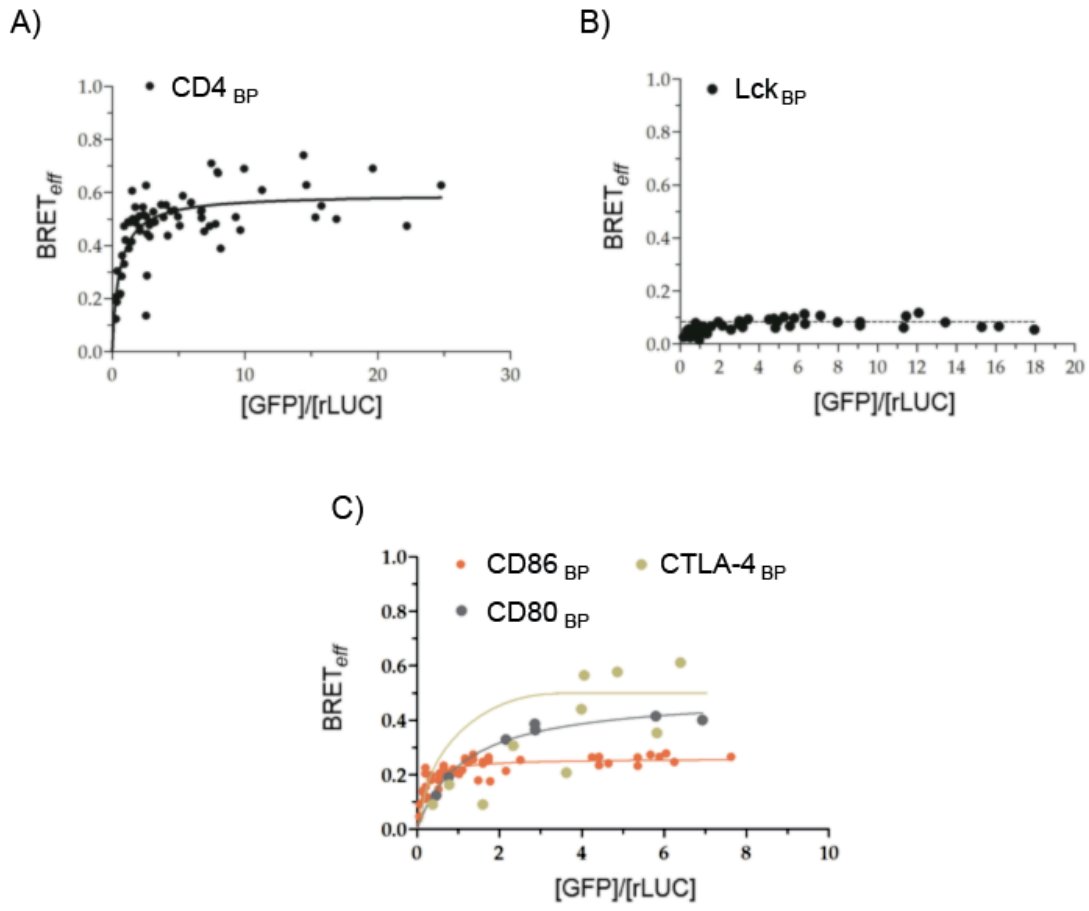

Figure 2 – BRET analysis of CD4 and Lck oligomerization levels. **(A)** Expression of full length CD4 as a BRET pair gives $BRET_{eff}$ values that are high and are a better fit to a trimer rather than dimer model, suggesting aggregation. (**B**) Expression of full length Lck as a BRET pair gives $BRET_{eff}$ values that are similar to monomers. (**C**) Standard BRET curves for molecules used as controls for monomeric (CD86), dimeric (CTLA4) and non-constitutive dimeric (CD80) interactions.

The co-receptor CD4 is functionally a monomer

Crystallization studies had demonstrated that CD4 can dimerize through its Ig-like domain 4 (d4) (343). To test whether the intracellular part of CD4 was responsible for the observed oligomerization, we generated a CD4 construct lacking the whole of the cytoplasmic tail, $CD4^{Ex}$, and tested it as a BRET pair (Fig. 3A). Compared with CD4 full length (Fig. 2A), $Bret_{eff}$ values for $CD4^{Ex}$ were reduced, displaying a monomeric protein profile. It thus became obvious that in our system the ability of CD4 to oligomerize was

dependent on its cytoplasmic tail. Initial work on CD4 recruitment of Lck to the vicinity of TCR upon T cell activation showed that four cysteine residues, two from CD4 and two from Lck, are responsible for the CD4-Lck interaction (194). It was also shown that the CD4 intracellular region forms an α-helix structure which can mediate binding to Lck (344), HIV protein Nef (345) and Vpu (346). For HIV interactions, CD4 cysteines are dispensable for binding, although the α-helix plays an important role (347). Because those intracellular cysteines, which are normally involved in Lck binding, could be mediating oligomerization, we mutated both to serine residues ($CD4^{\Delta Cys}$). A slight decrease in BRET efficiency was detected with the mutant but essentially the CD4 oligomerization was maintained (Fig. 3A).

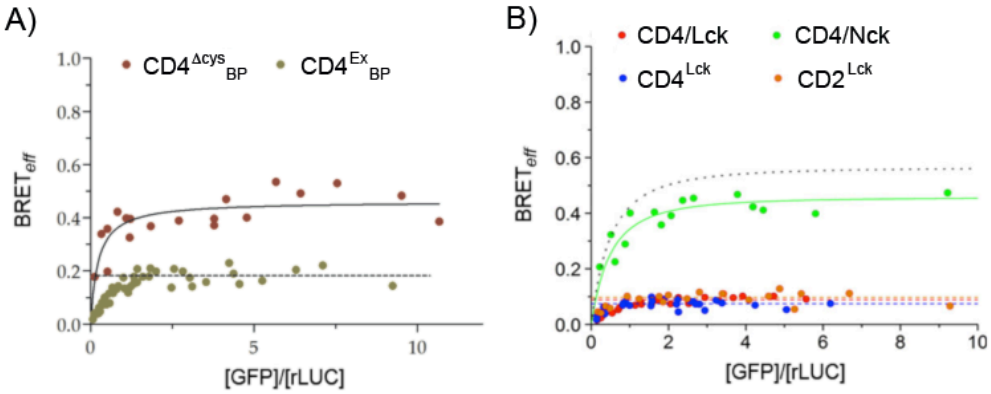

Figure 3 - CD4 is monomeric when Lck is present. (**A**) Mutation of the intracellular cysteine residues (ΔCys) lowers CD4 association but complete removal of the intracellular region ($CD4^{Ex}$) causes a large decrease in $BRET_{eff}$ values to levels observed only for monomeric proteins and shows independence from the acceptor:donor ratio. (**B**) Fusing the extracellular domain of CD4 to Lck ($CD4^{Lck}$ - blue), a construct that is known to function *in vivo* shows no self-association and gives $BRET_{eff}$ values that are identical to an equivalent construct using the CD2 extracellular domain ($CD2^{Lck}$ - orange). The same $BRET_{eff}$ values were observed when $CD4_{BP}$ was co-transfected with Lck labeled with mCherry (CD4/Lck - red). A control with CD4 and Nck (CD4/Nck – green) co-expression is also shown.

Since we had already ruled out the cysteine residues in primarily driving oligomerization, the amphipathic α-helix seemed the most likely candidate. We hypothesized that if this helix was causing oligomerization, then Lck binding to CD4 should abrogate it and cause dissociation. We co-expressed Lck (tagged with mCherry for visualization) in cells expressing the CD4 BRET pair and found that oligomerization was essentially abolished (Fig. 3B). This effect was specific to Lck, since co-expression of a control protein, myristoylated Nck1, did not produce a similar effect (Fig. 3B). The importance of this interaction in the context of the T cell was then established. Since the major role of CD4 in T cell activation is to bring bound Lck to regions of TCR–MHC

engagement, and the coreceptor is monomeric when bound to Lck, it must remain this way whilst carrying out its primary function. Littman and colleagues (348) showed that the interaction between the cytoplasmic tail of CD4 and Lck was an absolute requirement for the activation of T cells *in vivo.* The CD4 sequence fused to the full-length sequence of Lck ($CD4^{Lck}$) used in that study was here replicated and assessed for oligomerization. As this construct removes both the cytoplasmic α-helix as well as the conserved cysteines, it was anticipated not to show any evidence for association, and this was the case (Fig. 3B). To confirm this, an equivalent construct using CD2 in place of CD4 ($CD2^{Lck}$) was used, which gave values that were indistinguishable from those seen for the $CD4^{Lck}$ construct.

Lck is monovalent

Lck itself behaves as a monomer as seen in Fig. 2, although BRET values seemed very low compared with other monomers tested. What would then be the profile if we forced Lck to be a dimer? To answer this we made a chimera having the extracellular part of the constitutive dimer CTLA-4 and the full length Lck molecule (in the cytoplasmic part). This $CTLA\text{-}4^{Lck}$ construct showed higher BRET values than those obtained for Lck alone or the $CD4^{Lck}$ chimera, although they remained lower than those obtained for constitutive monomers (Fig. 4A). We wondered whether mutation of the Lck cysteines that mediate the interaction with CD4 could give the same effect as for the Lck BRET pair. Therefore, we tested $Lck^{C20/23}$ as a Bret pair as well as a chimera of $CD4^{LckC20/23}$. No significant differences could be identified when the BRET assay was employed in analogous way to wild type Lck (Fig. 4B). This suggests that essentially Lck behaves as a monomer in the context of T cell activation, which is in agreement with CD4 function. Improvements in the methodology (331) and in detection systems are nevertheless required to obtain more definitive conclusions. Given that the CD4-Lck chimera is capable of restoring T cell function in a $CD4^{-/-}$ mouse (348), it seems very likely that the functional complex of CD4 and Lck at the cell surface is monomeric.

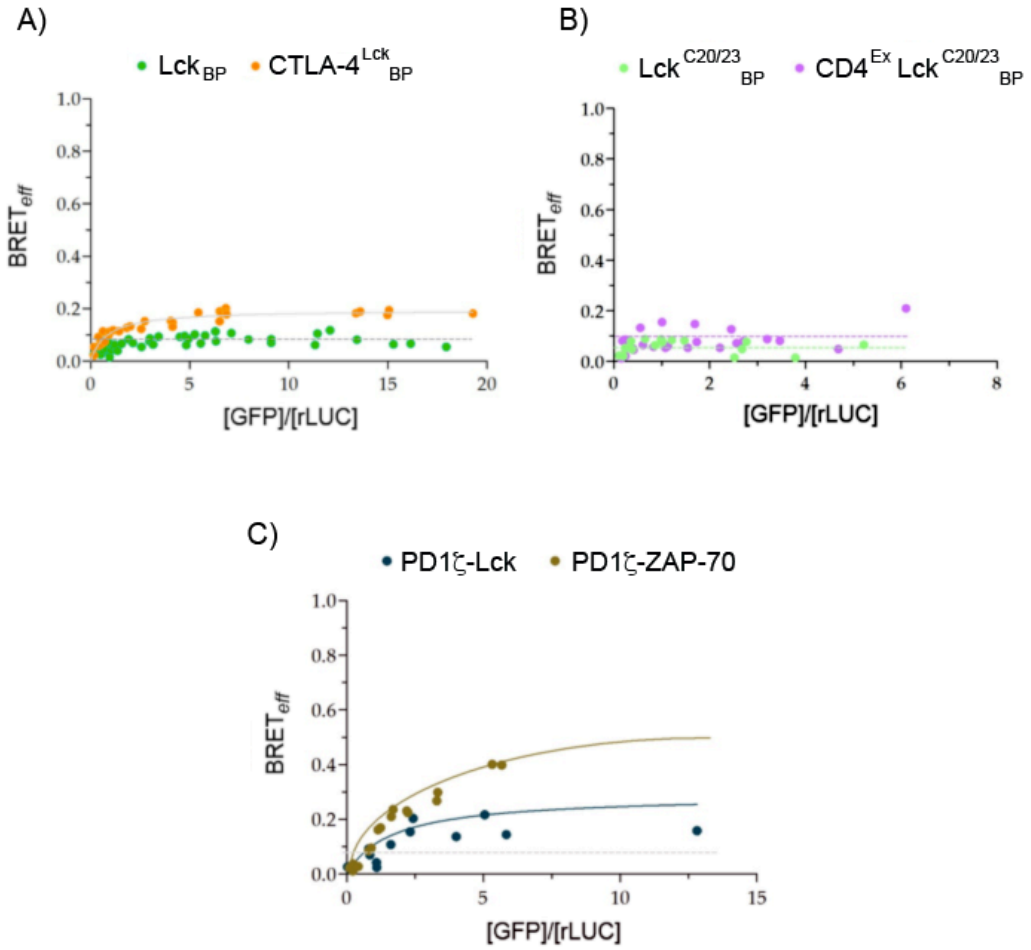

Figure 4 – Lck behaves as a monomer. (**A**) Bret analysis using Lck as a BRET pair (green) and the chimera of the extracellular part of CTLA-4 fused with full-length Lck (orange) suggests its monomeric behavior. (**B**) Lck having the CD4 binding cysteines mutated to serines (light green) and the chimera of this mutant with the extracellular part of CD4 (purple) also shows the same result. (**C**) BRET analysis of the chimeras of PD1 and CD3ζ with both Lck (blue) and ZAP-70 (brown) shows that the zeta subunit can associate with Lck.

One of the central events that occur after T cell activation is the recruitment of Lck to phosphorylate CD3ε, γ, δ and ζ chains of the TCR/CD3 complex (349). To assert if the lower $BRET_{eff}$ values compared with those normally associated with monomeric interactions are likely due to random interactions or because of large cytoplasmic domains of the constructs, we made a chimera expressing PD1 with the CD3 ζ chains and used with Lck and with ZAP-70, as a positive control, for BRET analysis. This shows that Lck can bind to ζ chains as it gave results higher than those obtained for Lck itself (Fig. 4C). Also, when we used PD1ζ-ZAP-70 we verified that the BRET values were even higher than for PD1ζ-Lck, indicative of a stronger interaction, as expected (Fig. 4C). These results demonstrate that the cytoplasmic tail is not the (only) cause for the lower values,

which can be due to a variable or deficient expression at the cell surface. Microscopy analysis showed that approximately 30% of these molecules remain inside the ER although the acceptor/donor ratio dependence of the data is unaffected.

Fyn kinase is a monomer at the cell surface

The most studied and predominant Src kinases expressed in T lymphocytes are Lck and Fyn, which share structural features. They are both localized at the membrane due to myristoylation/palmitoylation at the N-terminal region. In this N-terminus, also a “unique” domain for each protein is present and this is a major difference between both kinases and is critical for the specific functions of the proteins. The unique domain of Lck contains the two cysteine residues required for CD4 association, as mentioned earlier. However, the role of the unique domain of Fyn is not fully characterized, but it is known to be required for the subcellular localization of the protein (350). In order to characterize Fyn, we studied its stoichiometry using the BRET approach. We questioned whether Fyn was capable of oligomerization, and if so would this unique domain have some relevant role in the mechanism. BRET values for Fyn were very similar to those obtained for Lck (Fig. 5A), therefore we can conclude that Fyn, like Lck, is also a monomer in its functional state. After T cell engagement with pMHC, the most proximal signal events that occur are provided by Lck and Fyn functions (351). Although their roles in cellular activation are interdependent, they can have distinct functions along with this process. In both resting and activated cells, Fyn is associated with lipid rafts (328). Targeting of Lck to lipid rafts is dependent on the C-terminal region and may result in the formation of Lck-Fyn complexes (187, 352). Regarding this, we tested if using BRET we were able to identify this association. Our results showed that they could only randomly interact (Fig. 5B). We also tested the binding of Fyn with CD2, as it was demonstrated that these two proteins physically associate through the CD2 cytoplasmic tail (186). We could see that $BRET_{eff}$ values for this association are higher than those for Fyn_{BP} and similar to those obtained for $CTLA4^{Ex}Fyn_{BP}$, which could indicate that they can associate or that this association is probably specific in a given moment of T cell activation or in a specific site, like lipid rafts as mentioned earlier (Fig. 5C).

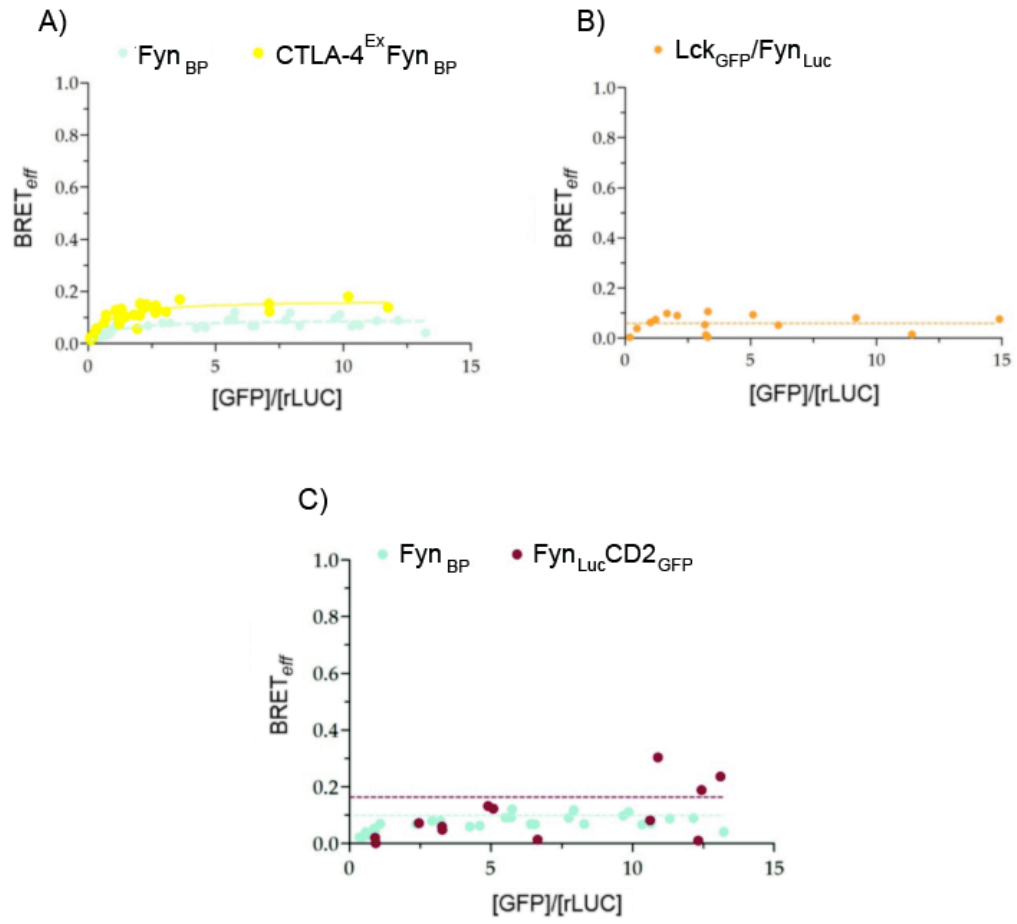

Figure 5 – Fyn behaves as a monomer. (**A**) Bret analysis using Fyn as a BRET pair (blue) and the chimera of the extracellular part of CTLA-4 fused with the full-length Fyn (yellow) shows its monomeric behavior. (**B**) $BRET_{eff}$ values for Fyn as a donor and Lck as an acceptor, showing no association between both proteins (orange). (**C**) Interaction analysis of CD2 with Fyn (purple) compared to Fyn_{BP}.

Association with Lck targets CD2 to lipid rafts

Previous work has shown that CD2 can also associate with Lck even in cells where CD4 or CD8 are absent (186) and that this association is mediated through the cytoplasmic tail of CD2, as mentioned for Fyn. Although human CD2 is not resident in lipid rafts (188), it was established that mouse and rat CD2 can associate with LRs (187, 353). To determine the molecular basis for the constitutive presence of a large fraction of rat CD2 in the rafts and, as binding to both kinases Lck and Fyn is mediated by the cytoplasmic domain, we sought to determine which segments could be playing a role. Previously characterized E6.1 Jurkat clones stably expressing cytoplasmic domain truncations of rat CD2 retaining 97, 81, 66, 40, and 6 aa of the cytoplasmic tail (46, 186,

336, 337) were used. The location of each mutant was analyzed following sucrose gradient centrifugation and immunoblotting of rat CD2 with OX-55. As shown in Fig. 6, the deletion mutants CD2(CY97), CD2(CY81), CD2(CY66), and CD2(CY40), like the full-length CD2 molecule, are mostly raft resident, whereas rat CD2(CY6) is totally excluded from lipid rafts and found in the soluble fractions.

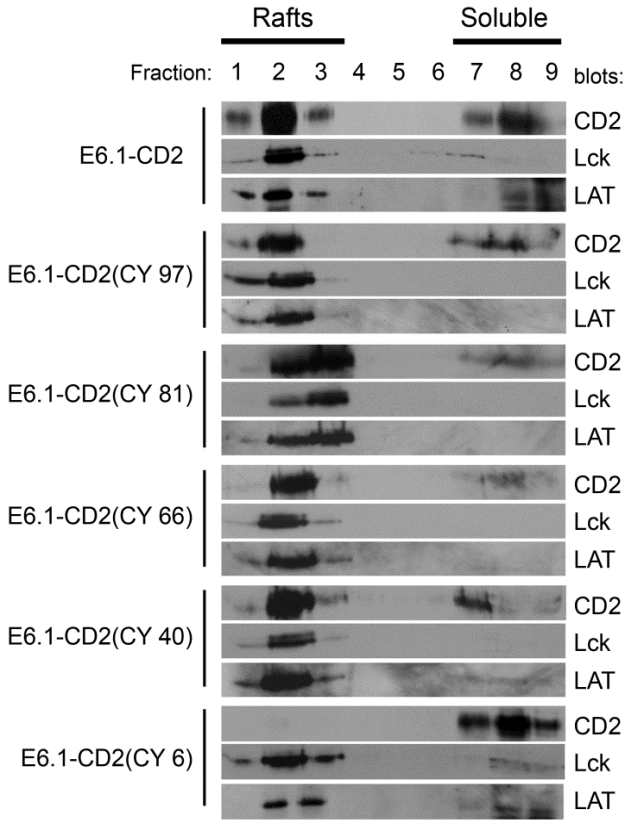

Figure 6 – CD2 mutants, except CD2-CY6, partition within lipid rafts. Fractions of sucrose gradient density centrifugation of lysates from E6.1 cells expressing rat CD2 cytoplasmic tail deletion mutants were immunoblotted for the localization of CD2, Lck, and LAT. Fractions recovered were numbered 1 to 9 from the top of the gradient. Cells analyzed were E6.1-CD2, E6.1-CD2(CY97), E6.1-CD2(CY81), E6.1-CD2(CY66), E6.1-CD2(CY40), and E6.1-CD2(CY6), expressing full-length CD2 and truncation mutants with the membrane proximal 97, 81, 66, 40, or 6 aa residues, respectively.

This finding suggests that the first 40 N-terminal amino acids of CD2 cytoplasmic tail are sufficient for the localization of CD2 in lipid rafts. As the pattern of association of rat CD2 truncation mutants with lipid rafts correlated entirely with the pattern of binding of these same mutants to the tyrosine kinase Lck (186), we raised the possibility that the interaction between rat CD2 and lipid rafts could be mediated via Lck. Moreover, as seen in Fig. 6, all CD2 mutants except CD2(CY6) are in general confined to the same fractions

as Lck. We tested further this hypothesis by constructing additional CD2 mutants that would have their association with Lck disrupted while retaining most of the cytoplasmic tail.

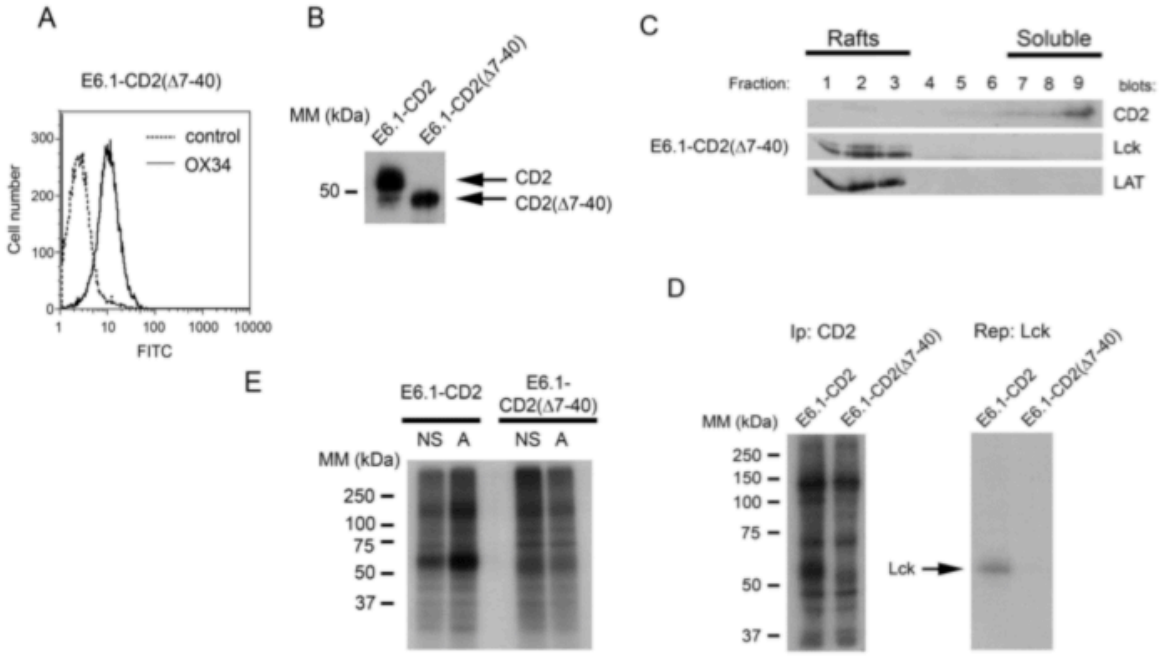

Figure 7 - The CD2 (Δ7–40) mutant looses the ability to bind to Lck, and does not target to lipid rafts. **(A)** Flow cytometry analysis of E6.1 Jurkat cells expressing rat CD2 with a deleted sequence between aa 7 and 40 of the cytoplasmic tail, CD2(Δ7–40). **(B)** Cell lysates from E6.1-CD2 and from E6.1-CD2(Δ7–40) cells were run on SDS-PAGE under reducing conditions, and immunoblotted using OX-55. CD2(Δ7–40) had the expected slightly lower molecular mass (MM) than wild-type CD2. **(C)** Fractions of sucrose gradient density centrifugation of lysates from E6.1-CD2(Δ7–40) cells immunoblotted with OX-55, Lck, and LAT demonstrate that CD2(Δ7–40) does not address to lipid rafts. **(D)** Immune complexes of CD2 from E6.1-CD2 and E6.1-CD2(Δ7–40) cells were subjected to *in vitro* kinase assays. Complexes were resolved by SDS-PAGE, and autoradiography of the dried gels (*left*) reveals several phosphorylated proteins in both lanes. A prominent 56-kDa phosphoprotein is clearly seen in the immunoprecipitates from E6.1-CD2 cells, but is absent from the immune complexes of E6.1-CD2(Δ7–40). Reprecipitation of Lck from the original complexes, with a polyclonal Ab, confirms the identity of the 56-kDa phosphoprotein from E6.1-CD2 lysates as Lck, still absent from E6.1-CD2(Δ7–40) reprecipitates (*right*). **(E)** E6.1-CD2 and E6.1-CD2(Δ7–40) cells were stimulated with CD2 mAb, following which cells were lysed and CD2 immunoprecipitated. Immune complexes were subjected to kinase assays and analyzed by SDS-PAGE and autoradiography. In E6.1-CD2 cells, Lck autophosphorylation increased sharply following cell stimulation, whereas in E6.1-CD2(Δ7–40) cells no major changes were detected. (NS – non stimulated cells; A – activated cells).

A mutant of the CD2 molecule excluding precisely the sequence between aa 7 and 40 of the cytoplasmic domain was tested. This new mutant, CD2(Δ7–40), was expressed at the surface of E6.1 cells, as detected by flow cytometry (Fig. 7A), and had an apparent molecular mass compatible with its slightly shorter tail (Fig. 7B). Strikingly, when we assessed the membrane localization of CD2(Δ7–40), we determined that this mutant does not localize within lipid rafts, but rather in the soluble fractions (Fig. 7C). We then tested whether CD2(Δ7–40) was incompetent for interacting with Lck, performing immunoprecipitations followed by kinase assays of the immune complexes of CD2 from E6.1-CD2(Δ7–40) cells, as well as from E6.1-CD2 cells for comparison. Among the proteins coprecipitated with CD2 from E6.1-CD2 cell lysates, a prominent phosphoprotein of 56 kDa was clearly visible, whereas no corresponding protein band was detected in the immune complexes from E6.1-CD2(Δ7–40) cells, although a number of other phosphoproteins with similar sizes are present (Fig. 7D). To confirm that the putative 56-kDa protein was indeed phosphorylated Lck, we performed a reprecipitation of Lck using a polyclonal Ab and confirmed the identity of p56 as Lck. No protein was detected in the reprecipitation of Lck from E6.1-CD2(Δ7–40) cells, suggesting that CD2(Δ7–40) looses the ability to bind to Lck, therefore strengthening our assumption that CD2 is trapped in lipid rafts fractions due to its association with the kinase. We checked whether having CD2 associated with Lck in rafts, as presented in E6.1-CD2 cells, would result in an increase of the Lck activity associated with CD2 in activated cells. In Fig. 7E, stimulation of CD2 with mAb triggers a sharp increase of the kinase activity associated with CD2 as shown by the increased autophosphorylation of Lck. By contrast, triggering CD2 in E6.1-CD2(Δ7–40) cells did not induce any detectable changes.

A physical association of rat CD2/Lck is sufficient for the raft targeting of CD2

To obtain definitive proof that Lck is the key determinant in the addressing of rat CD2 to lipid rafts, we resorted to the Lck-deficient J.CaM1 cellular model. Rat CD2 was stably expressed in J.CaM1 cells and its inclusion in the membrane fractions analyzed. Supporting our prediction, rat CD2 expressed in J.CaM1 cells localizes to the soluble fractions, whereas the kinase is absent in these cells as expected (Fig. 8A). Further evidence that Lck could couple rat CD2 to lipid rafts was obtained through co-capping experiments in which we induced capping of CD2.

Following fixation of the cells, we analyzed whether rafts would colocalize with polarized CD2 that would demonstrate a functional association between rafts and CD2. Rat CD2 labeled with OX-34-FITC could be visualized (Fig. 8B, green), and the

localization of rafts was detected by Alexa Fluor 568 (Fig. 8B, red) conjugated to Abs recognizing raft-resident LAT.

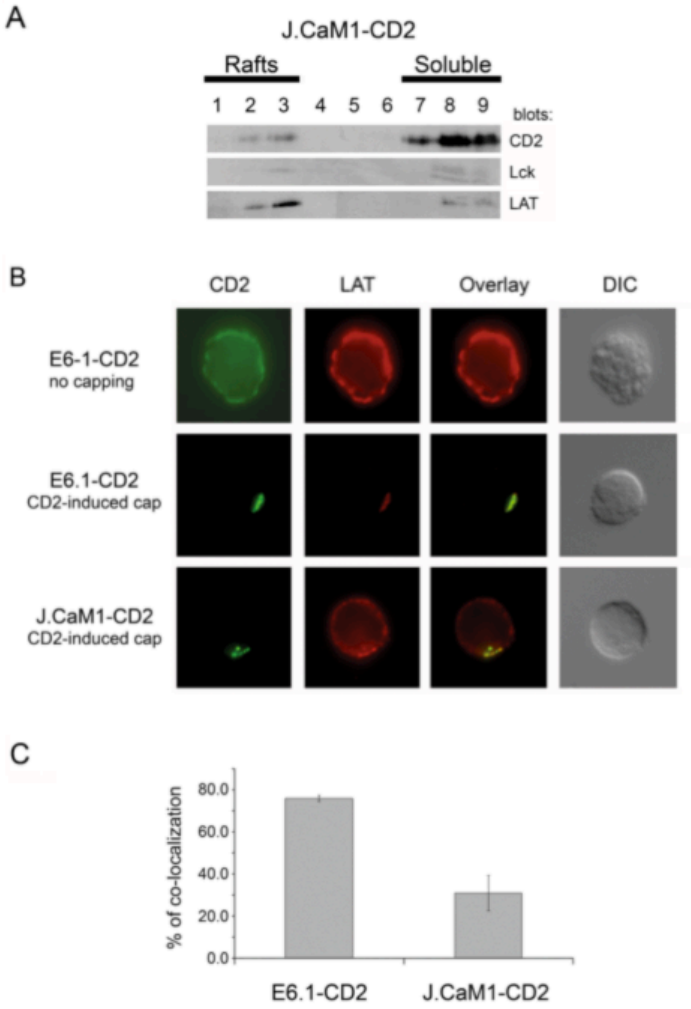

Figure 8 - Lck is required for the translocation of CD2 into lipid rafts. **(A)** Sucrose density centrifugation and immunoblotting with OX-55 confirm the exclusion of rat CD2 from the raft fractions of J.CaM1-CD2, an Lck-deficient cell line expressing rat CD2. Fractions were numbered 1 to 9 from the top of the gradient. Immunoblots of Lck attest the lack of expression of Lck, and LAT immunoblots confirm that these cells display normal raft behavior. **(B)** Analysis of co-capping of LAT with rat CD2 in E6.1-CD2 and J.CaM1-CD2 cells. Cells were incubated with FITC-conjugated OX-34 and allowed to cap for 15 min at 37°C. Following cell fixation, the raft-resident protein LAT was labeled with a specific serum. Cells were visualized by fluorescence microscopy, with CD2 caps detected in green, and LAT shown in red. The *top row* shows E6.1-CD2 cells had no capping induced. The differential interferential contrast (DIC) view is provided. **(C)** Quantitative analysis from two independent counts of a minimum of 200 cells from two different experiments show that LAT co-capped very neatly with CD2 in nearly 80% of E6.1-CD2 cells. By contrast, in J.CaM1-CD2 cells, co-capping was less well defined and detected in ~31% of cells. Data shown are mean percentage ± SE.

While in untreated cells no polarization of CD2 or rafts were induced (Fig. 8B, E6.1-CD2 cells, *upper row*), in E6.1-CD2 cells, capping of CD2 induced very clear co-capping

of LAT-labeled rafts in the majority of cells, nearly 80% (Fig. 8, B and C). By contrast, in the Lck-deficient J.CaM1-CD2 cells, capping of CD2 induced by CD2 Abs was not always accompanied by co-capping of LAT. In the fewer cells (31%) where we could observe LAT polarization, colocalization of LAT with CD2 was less well defined. Very similar results were obtained using co-capping of CD2 together with cholera toxin subunit B that binds to raft-embedded GM1 (data not shown).

Given that the J.CaM1 cell line is Lck deficient, we could use this cell line to address the raft function of CD2 in the absence of Lck if we could set up the conditions to artificially address CD2 to the membrane microdomains. We thus engineered a CD2 mutant containing the transmembrane and membrane-proximal intracellular sequences of the raft-resident adaptor PAG (212). This chimera was transfected in J.CaM1 cell line (Fig. 9A), showing a molecular weight of approximately 55 kDa (Fig. 9B). However, this mutant did not partition to lipid rafts in J.CaM1 cells, as shown in Fig. 9C. Conversely, this result further strengthens our claim that the CD2-Lck association is central to deliver CD2 to lipid rafts.

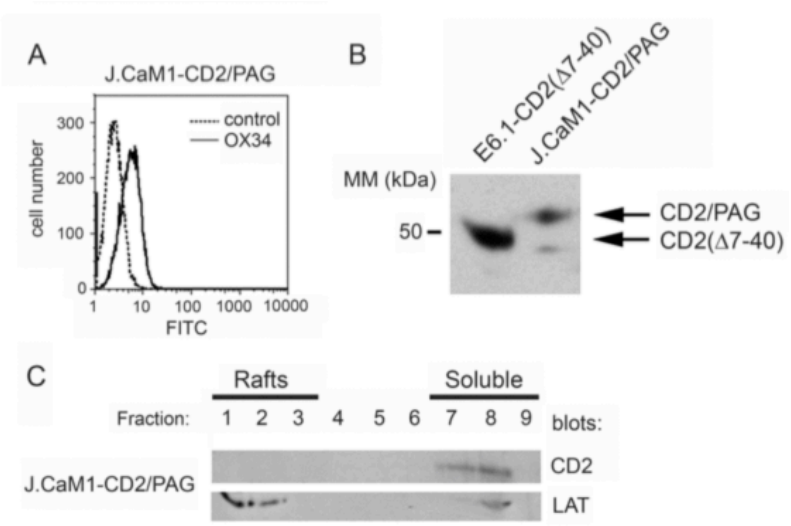

Figure 9 - A rat CD2/PAG chimera does not address to lipid rafts. A chimeric variant of CD2 with the transmembrane and cysteine motifs of PAG substituting the corresponding sequences of CD2 was expressed in J.CaM1 cells, as detected by FACS (**A**) and immunoblotting (**B**), with CD2(Δ7–40) shown for size comparison. (**C**) Sucrose density centrifugation and immunoblotting with OX-55 demonstrate that despite containing the raft-addressing motifs from PAG, the CD2/PAG chimera does not localize within rafts.

Discussion

For a proper T cell activation and signal transduction to occur, active kinases must be proximal to the TCR-pMHC complex. The initial goal of this study was to investigate the importance of the interaction of CD6 with Lck and with Fyn in its modulatory role in T cell activation, as we had demonstrated that both kinases associate with CD6. Although, it was not possible to use BRET for assaying CD6 interactions, we felt it was still relevant to investigate the organization and associations of the kinases Lck and Fyn at the cell surface. We thus used BRET analysis and biochemistry methods to pursue these goals.

Resonance energy transfer (RET) techniques have potential advantages to monitor protein-protein interactions in living cells and in real time. Among RET methods, BRET has been shown to allow the monitoring of intramolecular or intermolecular conformational changes with high sensitivity (354, 355). The delivery of Lck function in the proximity of TCR-pMHC is modeled by defined interactions with CD4 or CD8, providing phosphorylation of ITAMs present in the cytoplasmic region of the TCR-CD3 complex. To monitor Lck stoichiometry and Lck interaction with CD4, we fused both molecules with rLuc or GFP, acceptor and donor, respectively and performed BRET analysis. We showed that Lck alone had $BRET_{eff}$ values characteristic of a monomer, while CD4 alone showed to oligomerize having high $BRET_{eff}$ values, indicative of a higher order of oligomerization However when Lck was expressed as a chimera with CD4 or within the CD4 BRET pair, CD4 $BRET_{eff}$ values decreased to levels similar to Lck itself. This result indicates that functionally both Lck and CD4 are monomers. Nevertheless, it will be necessary to investigate further to clarify how both CD4 and Lck behave in T cells, where they are normally expressed.

The very low BRET values measured for Lck and Fyn could be due to the length of the molecules. As their size is considerably larger than any cytoplasmic tail of the transmembrane receptors assessed so far, oscillatory effects at the donor and acceptor parts could potentially diminish significantly energy transfer. To evaluate if the length of the proteins studied would affect the response detected, they were plotted against $BRET_{eff}$ values (Fig. 10).

When we compare proteins that have both extracellular and intracellular regions it is clear that there is no correlation between BRET values and the length, but rather with their multimeric organization; however, when kinases such as Fyn and Lck are subjected to BRET analysis, their size could be an issue. Proteins are fused to both rLuc and GFP at their N-terminal region which results in a quite long intracellular protein and this could affect the transfer of energy as some structural alterations can occur. Another explanation

for these lower values might be that most of these intracellular chimeras could be retained in the endoplasmic reticulum and not expressed at the membrane. However, for the CD4-FL construct, virtually all proteins reached the plasma membrane, and in the case of Lck-FL more than 90% of protein was also expressed at the membrane. Nevertheless, the $CTLA4^{Lck}$ chimera was present at the surface at only 58% of total protein, as observed by microscopy.

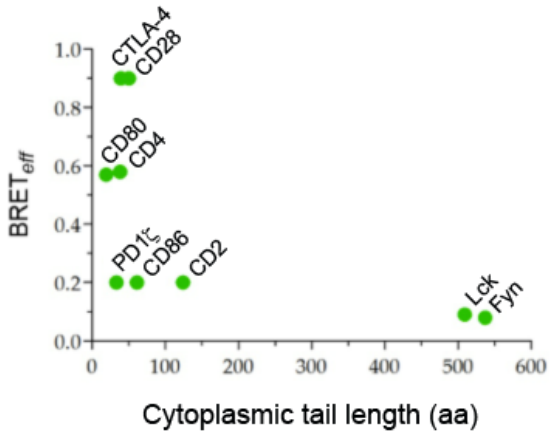

Figure 10 - Membrane protein cytoplasmic tail length and correlation with BRET signal. The calculated lengths of the intracellular regions of the proteins used in this study were plotted against the maximal BRET*eff* values determined for them.

Another important tyrosine kinase in T cell signaling is Fyn. Specifically, how this kinase is activated during T cell engagement is much less well understood than Lck. Whereas Lck binds to CD4 and CD8, Fyn can associate with CD3 subunits (198, 356). A study demonstrated that Lck might be directly involved in Fyn activation (352). When mouse cells were stimulated with CD4 or CD3, Lck activation correlated with its translocation to lipid rafts and consequently promoted the activation of Fyn. If Fyn could be bound to CD3 and Lck was the kinase responsible for the initial phosphorylation of both CD3ζ ITAMs and ZAP-70, would the kinases interact with each other? Previous evidence showed that Lck and Fyn can bind to TCR although with a low stoichiometry (198, 356). Our results show that nevertheless the association between the kinases can be detected, although at levels as low as those usually resulting from random interactions at the membrane level. Future work is required in order to understand the mechanisms of early and later Lck interactions when a whole synapse is formed.

Downstream responses of T lymphocytes after TCR activation are mediated by protein complexes that could be assembled in microdomains at the plasma membrane (357). This lateral segregation of plasma membrane into microdomains is named lipid

rafts (LR) (358). These are important platforms for signaling transduction to occur, and play a structural and significant role in temporal and spatial coordination of, for example, Lck and Fyn kinases (328, 359, 360). Both are targeted to the inner leaflet of the plasma membrane in LRs due to palmitoylation and myristoylation sites in their N-terminal region. Acylation is also required for Fyn interaction and phosphorylation of CD3ζ ITAMs (361). A study from Douglass and Vale (362) showed that the raft associated molecule LAT, as well as Lck, had a much larger diffusion coefficient than non-raft CD2, suggesting that lipid rafts can be highly mobile and diffuse to the TCR-activation spots. They also suggest that, as Lck and LAT change from a highly mobile to a motionless state when they encounter a signaling cluster, it is possible that protein-protein interactions, rather than any sort of raft-addressing labels, can play a major role in establishing specific interactions between raft and non-raft proteins. We demonstrate that the association of rat CD2 with membrane lipid rafts is in fact mostly determined by the physical interaction established with the protein tyrosine kinase Lck.

CD2 has been shown to associate to both Lck and Fyn protein tyrosine kinases (189, 330), and deletion of the cytoplasmic tail eliminates these interactions and abolishes CD2-mediated signaling (336, 363). Given that our results, using several mutant molecules of CD2 and Lck and different cells lines, show an absolute correlation between the capacity of rat CD2 to interact with raft-resident Lck and its ability to be found in lipid rafts, we can conclude that it is Lck, and not Fyn, that retains CD2 at the rafts in nonactivated cells. The interaction between Lck and CD2 has been extensively characterized, and it has been shown that a GST/Lck-SH3 fusion protein could bind, in solution, to peptides containing different proline-rich sequences of the cytoplasmic domain of rat CD2 (330). However, SH3-domain/proline-rich contacts do not fully account for the total of the CD2-Lck association, as the first 40 amino acids of the CD2 tail are enough for coprecipitating Lck (186). It was nevertheless unexpected that the removal of these amino acids would result in such a sharp decrease in the level of association (Figure 7, *D* and *E*), given that CD2(Δ7-40) still retains all proline-rich sequences. It is possible that the stretch of aa 6–40 of the CD2 cytoplasmic tail is crucial to set up the appropriate distance of the proline sequences to the SH3 domain of Lck, or to correctly orient the relevant modules. However, it is also plausible that this sequence, although functionally coupling CD2 to Lck, does not associate directly with Lck, and that other proteins or protein complexes mediate the CD2-Lck interaction at the membrane-proximal level and help directing CD2 localization. Thus, a more detailed analysis of the CD2 membrane-proximal sequence is required for establishing the key features and the functional consequences of the molecular interactions between CD2, Lck, and lipid rafts.

It is reasonable that following TCR-Ag recognition and CD2 engagement to its ligand and upon an encounter with a lipid raft, transitory interactions mediated through the CD2 ectodomain with a raft-resident receptor may allow for a transient overlap of the raft with CD2 complexes and induce raft-resident Lck to dock to the cytoplasmic domain of CD2. Association of CD2 and Lck is favored within lipid rafts. Nevertheless, the majority of CD2 and Lck are not resident in lipid rafts, neither in resting nor in activated cells.

What can then be the role of the two membrane proximal cysteine residues of rat CD2? It is intriguing that even using a chimeric CD2 molecule where we have inserted the relevant sequences of PAG, known to address very effectively the adaptor to lipid rafts (364), we failed to link CD2 to lipid rafts (Figure 8). Palmitoylation of integral proteins can be a very dynamic and reversible process, sometimes dependent on activation signals (365). For different transmembrane receptors, palmitoylation can occur at the plasma membrane or already in the endoplasmic reticulum (366). Thus, it is possible that CD2 is not available to lipid modifications depending on its own biosynthetic pathway, or that CD2 palmitoylation may depend on very specific activation signals, or even that the ectodomain may interact with other proteins that in some form can prevent the assembly of the complexes involved in lipid modifications to integral proteins. Alternatively, CD2 cysteines may perhaps help establishing protein-protein interaction with raft resident molecules, although it is unlikely that they mediate the actual association with Lck. The cysteine residues of Lck known to interact with CD4 and CD8 are localized further away from the membrane (344) and thus unable to contact to the CD2 membrane-juxtaposed residues. The determination of the function of these two amino acid residues will no doubt require further investigation.

Super resolution microscopy techniques such as PALM (Photoactivated localization microscopy) and STORM (Stochastic optical reconstruction microscopy) could also be used to study all those interactions mentioned in this study. These microscopy approaches analyze sequential activation and time-resolved localization of photoswitchable fluorophores to create high resolution images, in order to determine nano-scale interactions in different phases of T cell activation, thus helping to monitor and characterize stoichiometric and functionally the proteins involved in initiation and downstream signaling upon TCR-pMHC engagement.

Supplemental Methods

Constructs for BRET analysis

CD4 The CD4 gene was amplified using primers 1 and 2 from a full length construct and inserted into the BRET vectors as a *Pst*I/*Bam*HI fragment.

CD4Ex To remove the intracellular region of CD4, primers 4 and 5 were used to amplify CD4Ex, which was inserted as a *Mlu*I/*Bam*HI fragment into the BRET vectors.

Lck Cloned from a full length construct using primers 6 and 7 and inserted as a *Mlu*I/*Hind*III fragment into the BRET vectors. The GFP in pGFP2-N3 was first replaced by mCherry and then the Lck PCR product was inserted into this vector as a *Mlu*I/*Kpn*I fragment. Lck was fused to mCherry to facilitate quantitating its co-expression with the CD4 constructs.

Lck$^{C20/23}$Mutation of cysteines 20 and 23 of Lck were done with primers 16 and 17 in two rounds of PCR amplification. The same was done for Lck^{S6} construct but the 5' primer used was 18.

Fyn The Fyn gene was amplified from jurkat cell line cDNA with primers 8 and 9 and inserted in *Mlu*I/*Hind*III fragment into BRET vectors.

Nck As a control for Lck activity, Nck1 was cloned from a full length construct using primers 19 and 20. Primer 19 incorporated the first 8 amino acids of Lck at the N-terminus of Nck. The PCR product was then inserted as a MluI/BamHI fragment to replace Lck in the mCherry-fused vector.

CD4^{Ex}Lck CD4 was first amplified using primers 25 and 26 from the CD4WT GFP2 vector and cloned into the BRET vectors as a *Hind*III/*Kpn*I fragment. This removed the intracellular region of CD4. Lck was then amplified as a KpnI fragment using primers 27 and 28 and was inserted 3' of CD4^{Ex}.

CD2^{Ex}Lck CD2 was amplified from a full length construct using primers 23 and 24, which removed the intracellular region of the protein. The PstI/KpnI fragment of CD2Ex was then used to replace CD4 in the CD4Ex:Lck vector.

CTLA4^{Ex}Lck CTLA4 was amplified from a full length construct using primers 10 and 29, which removed the intracellular region of the protein. The *Hind*III/*Mlu*I fragment of CTLA4^{Ex} was inserted into BRET vectors. Lck was then amplified as a MluI fragment with primers 6 and 30 and inserted 3' of CTLA4^{Ex}.

Chapter III

GENERAL DISCUSSION

DISCUSSION

1. SRCR superfamily – search for new members

The Scavenger Receptor Cysteine-Rich (SRCR) superfamily comprises a collection of proteins that contain one or multiple domains structurally similar to the membrane distal domain of the type I scavenger receptor expressed by human macrophages (14). Proteins classified as belonging to this superfamily may include other types of domains in addition to the dominant SRCR modules, such as EGF, CUB, LCCL, or other domains. The SRCR superfamily is divided into two groups whose members have a few distinctive features: group A proteins are widely expressed from the most primitive phyla (echinoderms) to vertebrates, whereas group B members have only been found in vertebrates. Structurally, the distinction between the two groups is made by the number of exons that code for each domain (2 in group A and 1 in group B proteins) and the number of cysteines within individual SRCR domains (group A proteins have 6 cysteine residues that establish 3 disulfide bonds, whereas group B domains contain 8 cysteines and 4 bonds). Proteins within group B are characterized by having the SRCR domains near the N-terminal region and among mammalian species, group B orthologs are quite well conserved.

SRCR proteins are typically expressed in cells of the immune system (15), although some members can also be expressed in non-immune cells or organs, including liver, kidney, placenta, stomach, brain and heart (16). The immune role of many individual SRCR molecules has not been found yet, nor in fact has a unifying role for the family been established. We are especially interested in members belonging to the group B as our major goal is to identify and characterize important receptors that regulate immune responses.

By a comprehensive and systematic whole genome analysis, we have found a new putative transcript containing a cluster of 5 SRCR domains that we named SSc5D. This new SRCR protein has no transmembrane domain and was found to be mainly expressed in monocytes and T cells, whereas no expression on B cells was observed. As modeled in Figure 9 (Chapter II – Research Work - Section 1), the predicted dimensions of SSc5D are unusually big when compared with other group B members. Contributing to the size is the C-terminal region that very much resembles mucins. Overexpression and anomalous glycosylation of mucins can lead to cancer. Nevertheless, highly glycosylated proteins like SSc5D have different domains that can balance the degree of glycosylation as well as associate with other proteins in order to maintain homeostasis.

Comparison of the sequence of human SSc5D with that of mouse, rat and more primitive mammals (Platypus and opossum) orthologs, as seen in Figure 10 and 11 (Chapter II – Section 1), indicates that probably this gene was implicated in evolution of placental function, as functional gene expression of orthologs was not observed in monotremes or in marsupials. One of the possible functions advanced for SSc5D is that this protein detects pathogens and helps clearing them from these sites, perhaps also involving mucin-like attributes. SSc5D expression was detected in organs such as placenta, colon and lung, and in our current research we have determined that it is found mainly in epithelial layers.

CD5 and CD6 are probably the most well known members of the SRCR family, each containing three extracellular SRCR domains (21, 22). They associate with each other at the T cell surface (54, 84) and are implicated in the regulation of TCR activation and signaling. One of the best-characterized receptor-ligand interactions is that of CD6 binding to its ligand CD166, which is expressed at the APC surface (270). Alongside the CD6-CD166 interaction and the description of putative binding partners for CD5 (41-44), there was the initial concept that group B SRCR domains had a role mainly in intercellular contacts. In agreement, Spα (23) can interact with cells from myeloid and lymphoid origin. Regarding these three 3-SRCR domain-containing proteins it is possible to state that they can function in cell-cell communication, differentiation and activation. However, for the majority of the other members there are no counter receptors described or other associations that can help establishing a unifying function for this family. This lack of demonstration of cellular ligands raises the possibility that an altogether different function for SRCR domains may exist, if indeed SRCR domain proteins share any common function.

Our ongoing SPR-based analysis has demonstrated that the SSc5D N-terminal region is capable of binding to bacteria (Pereira *et al*, unpublished results). A recent study also showed that the mouse protein, essentially expressed in murine genitourinary and digestive tracts, can bind both bacteria and fungi (367). These data demonstrate the possible role of SSc5D in innate defense as well as homeostasis maintenance. Also, others have suggested that Spα (274), DMBT1 (275), CD163 (276), CD5 (277) and CD6 (83) can detect microbe-associated molecular patterns in order to clear bacteria or fungi, conferring a scavenger-like propriety to these molecules. Whereas a PRR function is not unexpected for soluble SRCR proteins, that same role attributed to T cell surface receptors was perhaps unforeseen. A deeper investigation into the pathogen binding

properties of CD5 and CD6 is thus needed to clarify whether these surface antigens may have dual functions in their overall immunological role.

2. CD6 an intriguing member of SRCR superfamily

CD6 has three SRCR domains that compose the N-terminal region. The membrane proximal SRCR domain (D3) is the one responsible for the ligand binding interaction with is counter-receptor CD166. The interaction of CD6 with CD166 results in both molecules being localized at the immunological synapse upon TCR triggering (84). Contrasting with CD5, there are no CD6 mouse models (knockouts, knockins or transgenics) that could really explain its function. Although much work has been done to elucidate the role of CD6, there are many researchers who maintain that CD6 is a co-stimulator of T cell responses, although some recent findings suggest otherwise.

The unusual large cytoplasmic tail of CD6 with 244 aa contains many signaling motifs, including nine tyrosine residues, two proline-rich regions, three PKC (R-R/K-x-S/T) and eight casein kinase II phosphorylation consensus sequences (SxxE/D), although only five of the latter are conserved between human, rat and mouse. The protein tyrosine kinases Lck, Fyn, Itk and ZAP-70 have been described to associate with rat CD6 through its cytoplasmic domain (54). Therefore, it may not be unexpected that when antibodies are employed to stimulate cells, CD6 is artificially aggregated with important signaling receptors that hold phosphorylation motifs, as are the cases of the TCR/CD3 complex or CD28. On the other hand, it cannot be excluded that CD6 may display a steady co-stimulatory effect, as seen by global intracellular phosphorylation and calcium mobilization (368-370). Nonetheless, when T cells are stimulated in a more physiological manner by antigen presenting cells, the expression of CD6 inversely correlates with the potency of the activation signals (Chapter II – Section 2 - Figure 1 and (67)). Moreover, microarray studies have shown a downregulation of CD6 expression in tumor-specific activated T cells (371).

Upon TCR triggering, CD6 is phosphorylated in Ser/Thr and also in Tyr residues (22, 70). Considering these observations and given that CD6 also associates with both Lck and Fyn, as seen in Figure 4C (Results - Chapter II – Section 2), how can this be reconciled with an inhibitory function? Suggestions to explain this could be given based on similar earlier studies on CD5. The surface glycoprotein CD5 is one of the first receptors to be tyrosine-phosphorylated upon TCR triggering (52), and yet it clearly inhibits T cell activation. The mode of CD5-mediated inhibition is not completely clarified,

but it probably involves the CD5 phosphorylation-dependent recruitment of the protein tyrosine phosphatase SHP-1 (45, 321), or alternatively the phosphorylation of the C-terminal inhibitory tyrosine residue of Fyn (55). Alternative or complementary mechanisms may include the functional association of CD5 with signaling auxiliary receptors such as CD2 (45, 46), which in turn intimately couples with the tyrosine kinases Lck and Fyn (186, 187). Given that CD6 also functionally interacts with CD2 and CD28 (70, 370), a similar mechanism where CD6 is phosphorylated by Lck and Fyn and in some way induces an inhibitory response can be considered. Alternatively, CD6 might be an activatory receptor, just like CD2, and equivalently provides the necessary signals to CD5 to put forth its inhibitory effect. Indeed, targeting CD6 directly with mAbs results almost exclusively in the phosphorylation of CD5, whereas cross-linking CD6 with the TCR induces strong phosphorylation in key signaling mediators like LAT (54). Knowing that CD5 and CD6 share common structural features, physically associate with each other, are both expressed at the surface of T cells and co-localize at the immune synapse established between T cells and APCs, one can hypothesize that they can be interdependently regulated and function together or in a way that they can compensate for each other's deficiency.

One additional factor that may influence the signaling nature of CD6 in different contexts is the variability of its cytoplasmic tail, which is dependent on alternative splicing regulation. These sequences are coded by different exons that can be differentially skipped. Despite some observations that in activated cells a different pattern of splicing is generated, studies to explore this potential mode of regulation of signaling through CD6 are still missing. Our analysis of tyrosine phosphorylation, IL-2 production and calcium fluxes of human CD6 mutants having different truncations of the cytoplasmic domain revealed that the region of CD6 which is critical for Ca^{2+} signaling is localized at the C-terminal end of the domain, shown by mutant CD6Cy179, in which the presence of Y572 constitutes the main difference from the preceding mutants (Figure 3B and 5A from results – Chapter II – Section 2). This result contrasts with those obtained by Kobarg *et al.* (71) where critical Ca^{2+} mobilization was mapped to the first half of the cytoplasmic tail. However, the kinase assay demonstrates that tyrosine phosphorylation is augmented in the mutant CD6Cy70, which comprises the first 4 tyrosines of the cytoplasmic domain. These tyrosines are presumably the target binding sites for SH2 domains of both Lck and Fyn. IL-2 assays correlate with the results obtained from the kinase assay, as the CD6Cy70 mutant already exhibited a significant reduction in IL-2 production. Yet, these data need to be explored further in order to define the exact mode of the CD6-mediated inhibitory mechanism.

3. Protein interactions and their stoichiometry at the plasma membrane

Having examined the signaling aspects of CD6, it seemed important to study the stoichiometry of binding of this scavenger receptor with signaling effectors. We thus took advantage of BRET, a powerful and sensitive technique that detects protein-protein interaction in living cells. This technology offers a higher efficiency and low background in comparison to other luminescence approaches. Although the BRET methodology is experimentally straightforward, there are several factors, such as distance, orientation and expression levels of the proteins of interest that could affect the efficiency of energy transfer. In our initial attempts to study CD6 stoichiometry with this method, we were faced with atypical BRET results. To understand this, the expression of CD6 at cell surface was scrutinized. By microscopy it was found that most of CD6 molecules were retained in the endoplasmic reticulum. Such result could be due to large oscillations occurring at the end of the long cytoplasmic tail of CD6, as BRET can only detect signals in ~10 nm distance. To clarify this issue, a tailess CD6 mutant was fused to both GFP and Rluc and subjected to BRET analysis. Also, no signals could be detected and the protein still remained in ER.

Despite these negative results and taking advantage of this technique we decided to go forward and study the proteins that somehow are connected to CD6 function, with particular focus on Lck and Fyn. Applying BRET analysis to both kinases, it was clear that they can only randomly interact between themselves and with each other as seen in Figures 3A, 4A and 4B (Results – Chapter II – Section 3). Although BRET data obtained for Lck gave results indicative of a monomer, others have postulated that the SH3 domains of Lck can dimerize (372). In that study, Lck dimerization occurs through Zn^{2+} ions, although the same Zn^{2+} ion "clasp" is present on CD4, meaning that cysteine residues that form this "clasp" are responsible for the association of Lck with CD4. In our study, CD4 dimerization was also observed, however this phenomenon only occurs when Lck is not expressed in the same cell as CD4. Essentially and regarding BRET*eff* obtained for CD4, Lck and Fyn, and others from additional studies, we could conclude that most of the molecules expressed at T cell surface are monomers in their active state. Although BRET might be an excellent method to investigate the stoichiometry of proteins expressed at the membrane, a significant fraction of Lck and Fyn expressed in T cells are not associated with the membrane. Elucidation on the organization of endogenous molecules requires further complementary analyses, such as the two color-coincident detection (TCCD) microscopy (373).

4. The relevance of protein interactions in the protein localization and association with membrane microdomains

Phosphorylation of the TCR by the tyrosine kinase Lck is a key event for the activation of T cells. Src kinases, such as Lck and Fyn, are involved in signal transduction downstream of T cell-associated receptors (374). Approximately 40% of the total Lck is constitutively active in a T cell, which proposes a tightly regulated mechanism to maintain the equilibrium between active and non-active Lck, in resting cells. The relative spatial and temporal distribution of receptors, kinases and phosphatases could explain this equilibrium (146). Association of CD6 and CD2 (186) with these phosphorylated kinases was also demonstrated in this work. How these receptors contribute to maintain Lck homeostasis is a daunting challenge that needs to be clarified. Membrane microdomains are small structural and functional units of the membrane confinement of signaling elements that are able to control enzymatic activities supporting T cell activation (324). Results from Chapter II - Section 3 show that the CD2-Lck interaction is required for CD2 to be targeted to lipid rafts. The LR concept is quite controversial: rafts were firstly described as small and heterogeneous signaling platforms, highly dynamic and enriched in cholesterol and sphingolipids (249, 375), whose main characteristic is to be resistant to solubilization by mild non-ionic detergents and are generally separated by a density sucrose gradient (376). Hence, the mode how LR are characterized casts serious doubts on their contents and even on their existence *in vivo* (377-379). Still, the recent state-of-art imaging techniques on live cells allowed the detection of such structures demonstrating lipid and protein dependent functional compartmentalization of the cell membrane (375, 380).

Super resolution microscopy techniques such as PALM (Photoactivated localization microscopy) and STORM (Stochastic optical reconstruction microscopy) could also be used to study all the interactions mentioned in this study. These microscopy approaches analyze sequential activation and time-resolved localization of photoswitchable fluorophores to create high resolution images, in order to determine nano-scale interactions on different phases of T cell activation, thus helping in monitoring and characterizing, functionally and stoichiometrically, the proteins involved in initiation and downstream signaling upon TCR-pMHC engagement. Indeed, a very recent paper uses these two approaches to show that Lck is clustered when it is active and prevents clustering in a close conformation state in a highly dynamic process (381).

Conclusion

The scavenger receptor cysteine-rich superfamily (SRCR-SF) constitutes an heterogeneous group of proteins, from soluble to membrane bound, expressed in lymphoid and non-lymphoid tissues and present in a broad spectrum of species. Important knowledge has been gained upon characterization of new members and through more thorough studies on the existing ones. Nevertheless, if there is an exact mechanism/function that can be assigned to these domains, it is still unknown. The great structural and phylogenetic conservation indicates that they have an important role in regulating the development and function of both the innate and adaptive immune systems. The major relevance of SRCR members is that they can be present in components of the natural barriers to infection, such as epithelia and macrophages, but are also expressed in lymphocytes, the major orchestrators of adaptive responses. The work in this thesis contributes with valuable insights on the evolution of the immune system, from innate to adaptive immunity.

Chapter IV

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